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The Structural and Functional Roles of Critical Amino Acid Residues in Drosophila Alcohol Dehydrogenase.

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THE STRUCTURAL AND FUNCTIONAL ROLES OF CRITICAL AMINO ACID RESIDUES IN *DROSOPHILA* ALCOHOL DEHYDROGENASE

A Dissertation

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In Memory of My Mother.

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ABSTRACT

Alcohol dehydrogenase (ADH, alcohol:NAD oxidoreductase, EC1.1.1.1) from *Drosophila melanogaster* (DmADH) is unique among alcohol dehydrogenases in metal ion requirement, substrate preference, and stereospecificity of hydride transfer. In order to study this unique enzyme, the first part of this dissertation reports the construction and expression of DmADH cDNA in *E. coli* hosts.

The second part of the dissertation shows the structural function of glycine-14 in putative NAD-binding domain of DmADH. The comparison of DmADH to horse liver ADH suggests that glycine-14 in DmADH is located at the first position of a "conserved sequence" which maintains the tight turn structure positioned in the NAD-binding domain. Mutating glycine 14 to valine virtually inactivates DmADH and alanine substitution causes a decrease in activity. Thermal denaturation, kinetic, and inhibition studies confirm that replacing glycine-14 with either alanine or valine leads to structural changes in the NAD-binding domain. These results provide direct evidence for the role played by glycine-14 in maintaining correct conformation in the NAD-binding domain.

The third part of the dissertation concerns two cysteinyl residues. DmADH is sensitive to DTNB which modifies one of the two cysteinyl residues in the enzyme. In order to identify the reactive cysteinyl residue and investigate its functional significance, one or both of the two cysteinyl residues has been changed to alanine (CA135, CA218 and CA135/CA218). None

of the mutants show decreased specific activity relative to wild-type, indicating that neither of the cysteinyl residues is essential for catalysis. CA135 and wild-type enzyme are both inactivated by DTNB, but CA218 and CA135/CA218 are unaffected by DTNB treatment, suggesting that DTNB introduces a steric interference at cysteine-218. DTNB modification of cysteine can be prevented by the substrates, NAD and 2-propanol, indicating that cysteine-218 may be in the vicinity of the active site. Cysteine-135 which is normally insensitive to DTNB becomes accessible in the presence of isopropanol and NAD, suggesting a conformational change induced by binding to these substrates.

CHAPTER ONE

INTRODUCTION

1.1 Background

Drosophila melanogaster alcohol dehydrogenase (DmADH) (alcohol:NAD oxidoreductase, EC1.1.1.1) reversibly catalyzes the conversion of alcohols to their oxidation products, aldehydes and ketone. In addition, several studies (Moxon *et al.*, 1985; Eisses *et al.*, 1985) have suggested that DmADH also uses acetaldehyde as a substrate for further oxidation. These facts are consistent with the physiological functions of DmADH in utilizing and detoxifying dietary alcohol. Since the major food of *Drosophila*, a fruit-fly, may contain high concentrations of alcohols, DmADH, therefore, is very abundant, forming up to 2% of total soluble protein in *Drosophila melanogaster*. DmADH is a small protein of 255 amino acid residues. It is a homo-dimer with a subunit molecular weight of approximately 27 KDa (Schwartz *et al.*, 1979; Thatcher & Sawyer, 1980).

DmADH has been the focus of a large research effort for over twenty years. In the mid-1960s, three laboratories (Grell *et al.*, 1965; Johnson & Denniston, 1964; and Ursprung & Leone, 1965) independently reported the discovery of ADH activity in extracts of *Drosophila melanogaster*. Four years later, the enzyme was purified by Sofer and Ursprung (1968). The *Dm**Adh*** gene was isolated by Maniatis *et al.* (1978) and Goldberg

(1980). Although gene isolation occurred ten years after the protein product was isolated, most of the current publications on DmADH relate to the structure and regulation of this gene. The structure of Dm*Adh* has been well established (Fig.1) (Benyajati *et al.*, 1981; 1983; Sofer & Martin, 1987). DmADH is encoded by a single gene containing two small introns (65 and 70 bp) within the coding region. However, the protein is translated from two different mRNAs, which are transcribed from two different promoters during fly development: the distal (adult) promoter and the proximal (embryonic-larval) promoter. As a result of this feature, DmADH activity rises slowly during larval life, falls to a very low level during pupation, and rises immediately to a new high level early in adult life (Savakis & Ashburner, 1985). DmADH expression shows not only timing but also tissue specificity. It has been determined that ADH activity is significantly higher in both larval and adult fat bodies than in other tissues. The *cis*-acting DNA elements necessary for correct developmental programming of Dm*Adh* are contained in the DNA region 5′ and 3′ to the coding sequence (Benyajati *et al.*, 1987; Fischer & Maniatis, 1988). The corresponding *trans*-acting factors have also been studied (Heberlein *et al.*, 1985).

Compared to the molecular biological studies of *Adh* gene and its expression, biochemical analyses of DmADH are relatively rare. Thus far, studies on structure-function relationships in DmADH have been based on the primary sequence of the protein. Most data used to address this issues were

derived from studies on natural DmADH variants and chemical or x-ray induced mutants. These approaches are limited by the facts that first, only a few ADH nulls are identified and characterized (see Table 2 for a list) and second, the enzymology of these proteins is understood only partially (Table 4) or not at all. Therefore, the objective of this dissertation is to investigate the structure-function relationships of DmADH by a new technique, site-directed mutagenesis.

1.2 Structural Properties of DmADH

1.2.1 Genetic Variation of DmADH and Chemical or X-ray Induced Mutants

One remarkable feature of DmADH is its biochemical polymorphism. Natural populations carry predominantly two electrophoretic forms (allozymes) of the enzyme: ADH-F and ADH-S (for fast and slow migration on SDS-PAGE). A list of *Adh* alleles known to encode active enzyme forms is given in Table 1 (Chambers, 1988; Ashburner, 1985). The variants listed show not only different electrophoretic mobilities on SDS-PAGE, but also different thermal stabilities and catalytic activities which will be discussed in Section 1.3.3. Amino acid sequencing of some variants indicates that only one or two amino acid differences in primary sequence exist, providing the useful probes to examine the structure-function relationship of DmADH.

Another way to locate critical structural portions for DmADH activity is to analyze ADH null (no activity) mutants generated by random mutagenesis (as distinguished from site-directed mutagenesis). Common methods used to induce mutants include x-radiation and chemicals such as ethyl methanesulfonate (EMS) and formaldehyde. A conditional lethal screening procedure for DmADH nulls invented by Sofer and colleagues (1972) makes the selection of mutants possible. *Drosophila* containing putative *Adh* null alleles are exposed to an unsaturated secondary alcohol. Such alcohols can be rapidly oxidized to highly toxic unsaturated ketones in ADH-positive flies. Thus, only those individuals lacking ADH activity can survive the selection. Over the past two decades, more than fifty DmADH nulls have been reported (Kelley *et al.*, 1985; Place *et al.*, 1987; Hollochor & Place, 1987; Lee, personal communication). Thirteen of these have been sequenced (Table 2). Since the amino acid substitutions induced by chemicals and x-radiation are random, studies based on these mutants are insufficient to determine their structural significance.

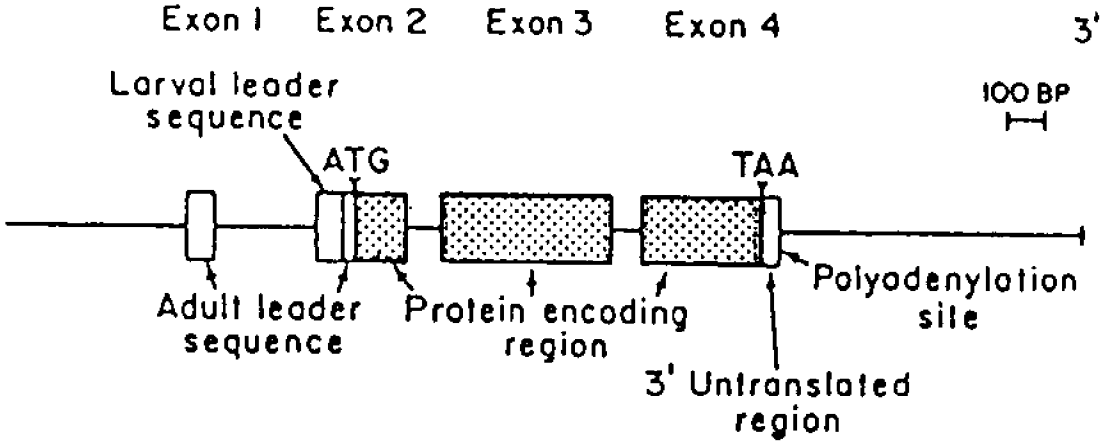


Fig. 1 **Diagram of the DmAdh locus showing introns, exons, larval and adult leader sequences, mRNA start site and poly A site as indicated (Kreitman, 1983).**

Table 1.

Allelic Variants of the *DmAdh* Gene

Variant	Description of phenotype	Source	Amino acid substitution (relative to ADH-S)[a]
ADH-US	Ultra-slow mobility variant	Africa[b]	—
ADH-Ss	Heat-sensitive enzyme with ADH-S mobility	N. America[c]	—
ADH-S	Standard ADH-S	Worldwide[d]	Standard
ADH-F	Standard ADH-F	Worldwide[d]	Lys 192 → Thr
ADH-Fr	Heat-stable enzyme with ADH-F mobility	N. America[c]	—
ADH-71K		Laboratory stock[f]	Lys 192 → Thr[e] Pro 214 → Ser
ADH-FCh.D		Australia[g]	Lys 192 → Thr[e] Pro 214 → Ser
ADH-Fs	Heat-sensitive enzyme with ADH-F mobility	N. America[c]	—
ADH-FR.City	Unstable enzyme	N. America[i]	—
ADH-F′	Stable enzyme with mobility slightly greater than ADH-F	Africa[h]	Ala 51 → Glu
ADH-UF	Ultra-fast mobility variant	Spain[j]	Asn 8 → Ala Ala 45 → Asp Lys 192 → Thr
ADH-D	Ultra-fast mobility mutant	Laboratory induced[k]	Lys 192 → Thr Gly 232 → Gly
ADH-A1	Recombinants	Laboratory cross[l]	—
ADH-B7			—

[a] After Ashburner (1985). References are also given in the text for amino acid replacement data.
[b] David *et al.* (1980).
[c] Sampsell (1977).
[d] Kreitman (1983).
[e] K. Th. Eisses (personal communication).
[f] Thörig *et al.* (1975).
[g] Gibson *et al.* (1980).
[h] Protein sequence data (Chambers *et al.*, 1981a) have been confirmed by DNA sequencing (C. Collet, personal communication).
[i] Fletcher (unpublished); see Chambers *et al.* (1984b).
[j] Malpica and Briscoe (unpublished); see Thatcher (1977).
[k] Grell *et al.* (1968).
[l] Maroni (1978).

* This table is adopted from Chambers (1988).

Table 2.

Missense and Deletion Mutations of *DmAdh*

Mutations	Mutagenic Agent	Position*	Sequence Changes	Ref.
Lee1	EMS	105	Thr - Ile	a
Lee2	EMS	117	Gly - Asp	a
Lee3	EMS	166	Ser - Phe	a
Lee4	EMS	168	Ala - Val	a
Lee5	EMS	168	Thr - Ile	a
Lee6	EMS	235	Trp - Arg	a
Adh^{n11}	EMS	14	Gly - Asp	b
Adh^{n8}	EMS	235	Trp - stop	c
Adh^{fn4}	Formaldehyde	Intron 2	17 bp deletion	d
Adh^{fn6}	Formaldehyde	Intron 2	6 bp deletion	d
Adh^{fn23}	Formaldehyde	241	34 bp deletion	d
Adh^{fn24}	Formaldehyde	63	11 bp deletion	d
Lee7	X-ray	183	Pro - Arg	a

[a] Lee, personal communication.

[b] Thatcher, 1980.

[c] Martin *et al.*, 1985.

[d] Benyajati *et al.*, 1983.

* Serine is counted as the No.1 residue.

1.2.2 DmADH Primary Sequence and A Predicted Secondary Structure

The primary sequence of DmADH has been fully determined (Thatcher, 1980) and confirmed by DNA sequencing (Goldberg, 1988; Benyajati *et al.*, 1980; 1981; Kreitman, 1983) (Appendix I). Comparing the primary sequence of DmADH with ADHs from other sources reveals no overall sequence homology. However, hypothetical secondary structure predictions using Chou-Fasman programs (1975) show an NAD-binding domain with a similar pattern to that of horse liver ADH (LADH) at the N-terminus of the DmADH molecule (Benyajati *et al.*, 1981; Thatcher & Sawyer, 1980) (Appendix II). The NAD-binding domain consists of alternating α-helixes and β-sheets (Fig. 2 & 3), a structural feature which is common to the NAD-binding domain of many NAD-dependent dehydrogenases (Rossmann, 1983). The N-terminal region of the NAD binding fold contains the sequence $GlyXGlyX_2Gly$ (X = any residue). This "conserved sequence" (Argos & Leberman, 1985) characteristically marks the tight turn following the first β-sheet of the NAD-binding domain in NAD-dependent dehydrogenases (Table 3). This "conserved sequence" has been reported in kinases (Walker *et al.*, 1982) and oncoproteins (Sternberg & Taylor, 1984) as well as in dehydrogenases.

In DmADH the first glycine residue in the "conserved sequence" is glycine-14. ADH isolated from the ADH^{n11} mutant (Section 1.2.1) contains an aspartic acid substitution at position 14. This mutant shows no ADH activity (Thatcher,

1980) and has a low affinity for NAD (Place *et al.*, 1979). It has been suggested that aspartic acid substitution probably disturbs the tight turn in the putative NAD-binding domain. However, it is unclear whether inactivation is due to the size or negative charge of the aspartic acid..

Little progress on structure-function analysis of DmADH has been made due to the lack of x-ray crystallographic studies on the protein. However, a recent review by Chambers (1988) reports that small ADH-F crystals have been isolated which show a threefold screw axis of symmetry.

Table 3.

Alignment of "Conserved Sequence" in NAD-dependent Dehydrogenase

Protein	Residues	Sequences
GAPDH	1-20	SKIGIDGFGRIGRLVLRAAL
LDH	21-40	NKITVVGVGAVGMACAISIL
LADH	193-212	STCAVFGLGGVGLSVIMGCK
DmADH	8-27	NVIFVAGLGGIGLDTSKELL

GAPDH: Glyceraldehyde-3-phosphate; LDH: Lactate dehydrogenase; LADH: Liver alcohol dehydrogenase; DmADH: Drosophila alcohol dehydrogenase.

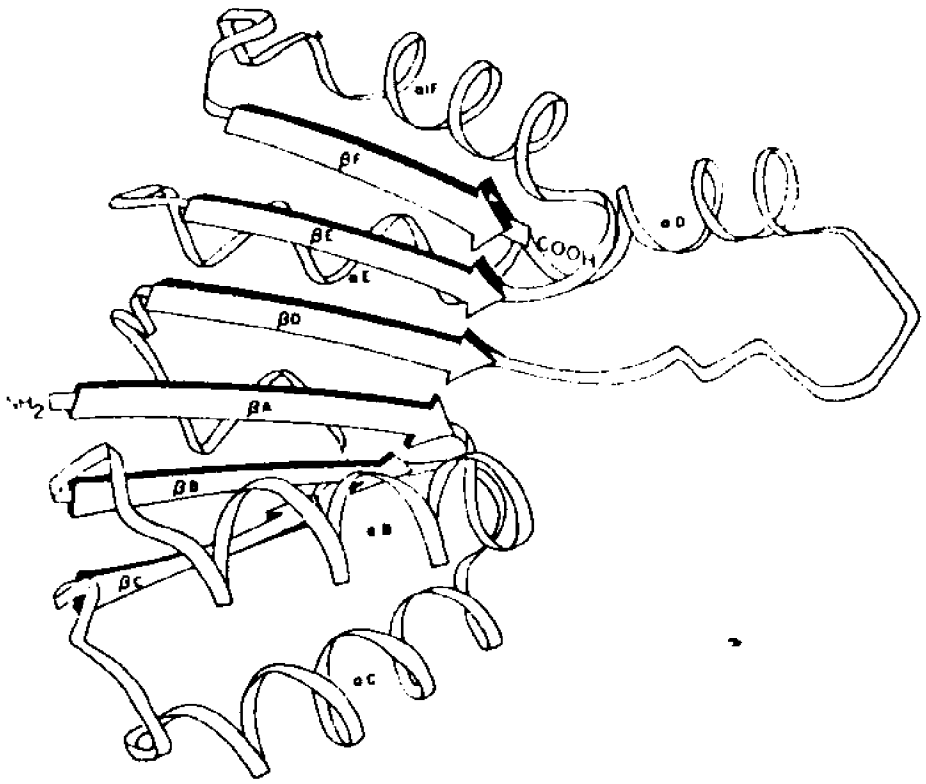


Fig.2 **The NAD-binding Domain Common to NAD-dependent Dehydrogenases (Rossmann, 1983).**

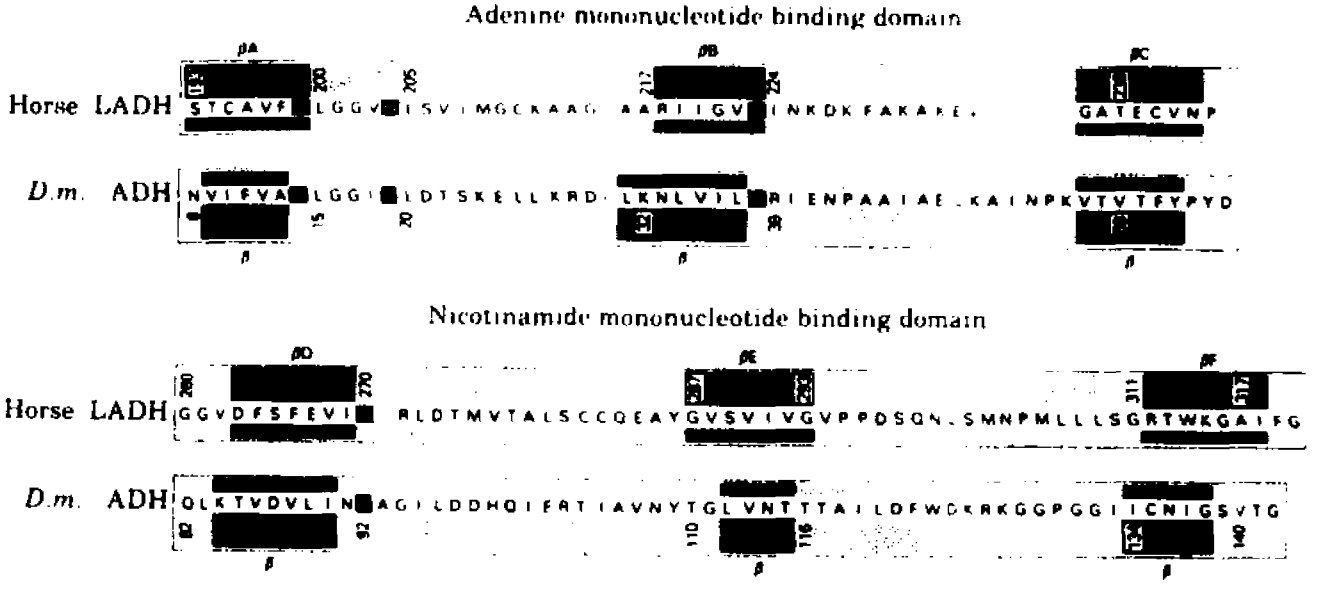


Fig.3 **Comparison of NAD-binding Domain of Horse LADH with the Predicted NAD-binding Region of DmADH (Benyajati et al., 1981).**

1.2.3 Structure-function Analysis from Isozyme Convention and Chemical Modification

Electrophoresis of total protein extracts of homozygous flies of any *Adh* genotype produces two or three isozymic staining regions (ADH-5, ADH-3, and ADH-1, in the order of anodal migration). The origin of these forms is well understood. They arise from the binding of zero, one, or two molecules of an NAD-carbonyl adduct per ADH dimer (Schwartz *et al.*, 1979 and Winberg *et al.* 1988a). By using gel electrophoresis in the presence of coenzyme, Winberg *et al.* (1983) studied isozyme conversion in pure ADH-S (slow allele). They found that at least one of the multiple forms of purified DmADH appeared during purification. This enzyme fraction can not be converted into an NAD-carbonyl containing enzyme, even if incubated with NAD and acetone. It is probably a deamidated form of DmADH as suggested by Thatcher (1980). At least one of labile amides in DmADH is located in the coenzyme binding region which prevents NAD binding.

In addition to studying the function of the labile amides in DmADH, two cysteinyl residues have been examined by chemical modification. In general, cysteinyl residues are considered to be potential catalytic site in enzymes because of the nucleophilic nature of the sulfhydryl group. In previous studies on DmADH (Thatcher, 1981 and Chambers *et al.*, 1981), it has been found that this dimeric enzyme contains two cysteinyl residues per monomer, cysteine-135 and cysteine-218. In the native enzyme, one cysteinyl residue reacts rapidly with 5,5'-

dithiobis(2-nitrobenzoate) (DTNB) causing a loss of ADH activity. The other cysteinyl residue is almost unreactive toward DTNB in the native enzyme. Chambers *et al.* (1981) suggested that cysteine-135 was the DTNB-reactive residue and found that after chemical modification by iodoacetamide , most of cysteine-218 was recovered as an unmodified cysteine. However, cysteine-135 was never recovered from modified enzyme for a conclusive identification because this residue was located in a large hydrophobic peptide region. On the other hand, Thatcher (1981) indicated that this enzyme was resistant to alkylation with iodoacetic acid or iodoacetamide. Therefore, chemical modification alone has been insufficient to determine the function and to identify of the cysteine which is sensitive to DTNB.

1.2.4 Structural Information from Substrate Specificity and Inhibition Behaviors

X-ray crystallographic studies of LADH suggest that the alcohol binding site contains one hydrophobic funnel to which the alkyl group of the primary alcohol binds preferentially (Eklund *et al.*, 1976). This structure has also been suggested for yeast ADH which, in contrast to LADH, is more active toward ethanol than larger alcohol such as n-butanol. This indicates the existence of a larger hydrophobic funnel in LADH than in yeast ADH.

On the other hand, DmADH is quite different from LADH and yeast ADH. It prefers secondary alcohols as substrates (Sofer

& Ursprung, 1968; Winberg *et al.*, 1982a). The difference in substrate specificity between DmADH and LADH or yeast ADH indicates that there are substantial differences in the alcohol-binding sites. Its high activity toward secondary alcohol suggests that DmADH may bind both alkyl groups of the substrate through hydrophobic residues. In substrate specificity studies, identical Vm and Km values for the two enantiomers of 2-butanol support this idea. If the hydrophobic interaction between enzyme and substrate involved only one of the two alkyl groups of 2-butanol, as in LADH or yeast ADH, only the ethyl group in one enantiomer or the methyl group of the other should bind to the hydrophobic site of the enzyme. In such a case, large differences in Vm/Km between the two enantiomers should be observed; this is not the case in DmADH.

In inhibition studies, chemical compounds which are known to be good inhibitors of LADH have been used with DmADH (Winberg *et al.*, 1982b). Salicylate is a coenzyme-competitive inhibitor of dehydrogenases (Dawkins *et al.*, 1967). It binds to the hydrophobic pocket at the adenosine part of NAD, but not to the zinc binding moiety (Einarsson *et al.*, 1974) in LADH. Salicylate is also an NAD-competitive inhibitor of DmADH. The Ki value of salicylate for DmADH is almost identical to that for LADH (Dawkins, 1967), suggesting a similar adenosine binding pocket.

1.3 Functional Properties of DmADH

1.3.1 Major Functional Differences between DmADH and Other ADHs

DmADH shows not only little homology in primary sequence but also many functional differences when compared to the corresponding enzymes obtained from mammals, maize, and yeast. In addition to the differences in substrate preference as discussed in Section 1.2.4, DmADH requires no metal cofactors (Chambers, 1984), and stereochemically shows 4-pro-S hydride transfer (Benner *et al.*, 1985). Horse liver and yeast ADH, in contrast, are more specific for primary alcohols (Dalziel & Dickinson, 1966), require zinc ions as cofactors (Dunn & Hutchinson, 1973), and show the 4-pro-R stereospecificity (You, 1982).

1.3.2 Reaction Mechanism of DmADH

The Theorell-Chance mechanism (Theorell & Chance, 1951) has been suggested for DmADH when secondary alcohols are used as the substrate (Winberg *et al.*, 1982a). According to this mechanism, ternary complexes are not kinetically significant and the dissociation of enzyme-NADH complex is the rate-limiting step. Several lines of evidence have supported this mechanism: (i) for many of the secondary alcohols tested, Vm is essentially the same (Winberg *et al.*, 1982a); (ii) deuterated secondary alcohols do not give primary kinetic isotope effects (Heinstra *et al.*, 1988); and (iii) product inhibition displays the Theorell-Chance reaction feature (Heinstra *et al.*, 1988).

With primary alcohols, in contrast of the above, the Vm of DmADH increases from ethanol to n-butanol, then decreases from n-butanol to n-octanol. The Vm for primary alcohols are much lower than those of corresponding secondary alcohol following Theorell-Chance mechanism. A primary kinetic isotope effect of around 3 occurred with deuterated ethanol. Furthermore, no product inhibition appeared at high ethanol concentration. This indicates that with primary alcohols, ternary-complex interconversion, i.e. hydride transfer, becomes the rate-limiting step. Winberg *et al.* (1982a) suggested that with primary alcohols, DmADH behaves similar to a carboxymethyl LADH (Reynolds *et al.*, 1975), in which the rate-limiting step is the interconversion of the ternary complex. However, LADH normally follows the Theorell-Chance mechanism for primary alcohols with the dissociation of enzyme-NADH complex as the rate-limiting step.

1.3.3 **Kinetic Constants and Thermal Stability of DmADH**

DmADH is not an allosteric enzyme. It obeys typical Michaelis-Menten kinetics. Table 4 lists the Km and specific activity relative to ADH-S for several DmADH variants.

Km's for ADH-F and ADH-uF are more or less the same and both are higher than that of ADH-S and ADH-71K (Table 4), but the rank order of catalytic activity is ADH-uF > ADH-F > ADH-71K > ADH-S. It has been reported that ADH-71K does not show hydride transfer as the rate-limiting step for primary alcohol

catalysis (Heinstra *et al.*, 1988). As discussed in Section 1.2.1, the difference of amino acid sequence between the ADH-S and ADH-F allozymes of DmADH is in the amino acid at position 192 (Thatcher, 1980); between ADH-71K and ADH-F, in amino acid at position 214 (Heinstra, 1987 and Heinstra *et. al.*, 1988); between ADH-uF and ADH-F, differences are found at both position 8 and 45. Thus, replacements in N- or C- terminal regions seem to influence enzyme function in an unpredictable manner. Viewing the DmADH null data (Section 1.2.1) with respect to these facts suggests that the entire tertiary structure of DmADH is involved in binding substrate and coenzyme (Hovik *et al.*, 1984).

On the other hand, the rank order of thermal stability of the above allozymes is ADH-71K > ADH-F > ADH-S > ADH-uF (Chambers *et al.*, 1984), supporting the premise that a single amino acid substitution may cause structural variation in DmADH.

Table 4.

Km and Relative Specific Activity for DmADH Isozymes

Enzyme	[a]Km (mM)		[b]Specific Activity (relative to ADH-S)	
	Ethanol	2-propanol	Ethanol	2-propanol
ADH-S	3.70	0.56	1.0	1.0
ADH-71K	4.5	N.A.	1.4	1.5
ADH-F	6.54	1.08	2.4	3.8
ADH-uF	5.5	1.2	7.5	4.5

[a] Km values are measured in pH 8.5-9.5 at room temperature and referenced from Winberg *et al.*, 1982, 1988b; Chambers, 1984; Heinstra *et al.*, 1988.

[b] Data are from Chambers *et al.*, 1984.

1.4 Site-directed Mutagenesis and Protein Engineering

Reviewing the past two decades of DmADH studies, it can be seen that neither natural variants nor randomly induced mutants have provided sufficient information to determine the structure-function properties of this enzyme. In order to further study these important properties for DmADH, individual amino acid residues must be examined for their structural and functional roles. Therefore, site-directed mutagenesis becomes a very powerful tool to achieve this goal.

The fundamentals of site-directed mutagenesis were developed in the early 1970s (Hutchinson & Edgell, 1971), but it was not until the late 1970s that the maturation in the techniques of oligonucleotide synthesis led to wide-spread application of site-directed mutagenesis for producing single or multiple base substitutions, insertions and deletions. The methodologies and applications of this technology have been reviewed (Smith, 1985; Craik 1985; Carter, *et al.*, 1985; Carter, 1986; Leatherbarrow & Fersht, 1986).

Classically, site-directed mutagenesis involves [i] annealing a mutagenic oligonucleotide primer to a single-stranded DNA template, [ii] *in vitro* synthesizing the second strand from the synthetic primer by *in vitro* DNA polymerization and ligation, and [iii] transforming the produced double stranded DNA into a bacterial host. More recently, polymerase chain reaction (PCR) (Higuchi *et al.*, 1988) has been used to generate predetermined mutations. The advantage of this technique is that either double-stranded or single-stranded DNA

template can be used, and mutations can be created *in vitro* without transformation. Both techniques have been used in this dissertation.

A key problem in site-directed mutagenesis is the low mutation rates. A common strategy to enhancement the frequency of mutations is to create an asymmetry between the two DNA strands by means such as hemimethylated DNA (Kramer *et al.*, 1982; Marmenout *et al.*, 1984), nonsense codons (Kramer *et al.*, 1984), the EcoK/EcoB system (Carter *et al.*, 1985), γ-phosphorothionate dNTP analogues (Taylor *et al.*, 1985a,b), and uracil containing DNA strand (Kunkel, 1985; and Kunkel *et al.*, 1987). The mutation can be enriched by either physical separation or genetic selection.

Kunkel's procedure (1985) for mutation enhancement is used in this dissertation. Briefly, a single stranded uracil-containing M13 template is prepared in a dUTPase and uracil N-glycosylase defective *E. coli* strain. The second strand, synthesized *in vitro* from a mutagenic primer, does not contain uridine. Transformation of the heteroduplex into an *E. coli* strain wild-type in dUTPase and uracil N-glycosylase results in excision of uridine so that the strand which carries the mutation is preferentially enriched.

Site-directed mutagenesis was first applied to study protein function to tyrosyl-tRNA synthetase (Winter *et al.*, 1982), β-lactamase (Dalbaldie-McFarland *et al.*, 1982; Sigal *et al.*, 1982; and Charles *et al.*, 1982) and prolipoprotein (Inouye *et al.*, 1982). In recent years, protein engineering has been

widely applied to alter the thermal stability of a protein (Nicholson, *et al.*, 1988; and Kellis Jr, *et al.*, 1988); change substrate-specificity (Wells *et al.*, 1987; and Bone *et al.*, 1989); and study peptide folding pathways (Oas & Kim, 1988). The most common application of protein engineering by site-directed mutagenesis is for studying the catalytic and regulatory mechanisms of enzymes; specifically, how an individual amino acid residue contributes to enzymatic function. For example, in serine proteases, three key amino acid residues (a catalytic triad) have been mutated systematically to determine their function (Craik *et al.*, 1987; Sprang *et al.*, 1987). Fructose 6-phosphate-1-kinase (PFK), an allosteric enzyme, has been mutated at its regulatory sites to investigate the conversion between tight and relaxed conformation as well as regulation by individual ligand (Valdez *et al.*, 1989). It is, therefore, possible to elucidate the structure-function relationship of a enzyme by protein engineering and site-directed mutagenesis.

1.5 **Rationale and Objectives**

The physical and biological properties of a protein molecule are determined by its structure. In nature, proteins with related functions usually show common structural features. The enzymes in the serine or cysteine protease families, for instance, have three common residues (a catalytic triad) in their active site (Fersht, 1985). Some DNA binding proteins

contain zinc fingers or leucine-zippers for dimerization (Klug & Rhodes, 1987; and Landschulz *et al.*, 1988).

DmADH is unique among alcohol dehydrogenases in its metal ion requirement, substrate preference, and stereospecificity of hydride transfer. However, the structure-function relationship has not been elucidated. There are two basic questions remaining on DmADH studies.

First, how closely related, structurally, is DmADH to other alcohol dehydrogenases? NAD-dependent dehydrogenase have been shown to have a common NAD binding domain of six parallel β-sheets connected by α-helixes (Rossman *et al.*, 1975; 1983). Although DmADH has no overall sequence homology with LADH or other ADHs, a predicted secondary structure reveals a similar NAD binding domain and several highly conserved residues (Benyajati *et al.*, 1981). On the basis of this speculated secondary structure, experiments were designed to investigate the function of this predicted NAD-binding domain. Results from this study support the notion that common structural features may play an important role in the coenzyme-binding of NAD-dependent dehydrogenases, including DmADH.

The second question concerns the catalytic mechanism of this unique ADH. It has been shown that the substrate binding site of DmADH is adjectively different from that of LADH, and that there is no zinc ion involved in the catalytic reaction (Section 1.2.4 & 1.3.1). LADH has several cysteine residues which serve to hold the zinc required for the catalytic activity. But, DmADH has only two cysteines and, more

importantly, no zinc ion. One of the cysteines shows sensitivity to chemical modification with an accompanying loss of ADH activity. The function and position of these two cysteines in the catalytic fold are unclear. Clarification of earlier ambiguous observations would help locate the active site and, further, deduce the reaction mechanism of DmADH which might be different from that of LADH.

DmADH has a small coding gene with two short introns. It has been cloned into the M13mp18 vector in our lab. By using site-directed mutagenesis, it is possible to manipulate this gene for its expression in *E. coli* host and to change certain key amino acids for the structure-functional investigation of DmADH.

The specific aims of this study are the following:

1. To construct a DmADH cDNA and establish a high expression system for DmADH.

2. To test the structural role of glycine-14 in a putative NAD-binding site of DmADH; therefore, to prove a common NAD-binding domain for DmADH with other ADHs.

3. To test the function of the two cysteine residues in DmADH and to locate the active site of this enzyme.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Enzymes and Antibody

All restriction endonucleases, DNA ligase, DNA kinase, mung bean nuclease, T4 DNA polymerase, and Klenow DNA polymerase were purchased from Bethesda Research Laboratories (BRL) (Bethesda, MD), New England Biolabs, Inc. (NEB) (Beverly, MA), Promega Biotec Corporation (Madison, WI), Boehringer-Mannheim Biochemicals (Indianapolis, IN), and Pharmacia (Piscataway, NJ). Nick translation kits and ligation kits were ordered from BRL. Sequenase kits were obtained from United States Biochemicals Corp. (USB) (Cleveland, OH). *Taq* DNA polymerase kits which were used for polymerase chain reaction (PCR) were from Perkin Elmer Cetus (Norwalk, CT). Pancreatic ribonuclease and deoxyribonuclease were bought from Sigma Chemical Co. (Sigma) (St. Louis, MO). Alcohol dehydrogenase isolated from *Drosophila melanogaster* and rabbit antibodies against the fly protein were prepared by Batzer *et al.* (1988).

2.1.2 Chemicals

Bacto-tryptone, bacto-yeast, and bacto-agar were products of Difco Laboratories (Detroit, MI). Agarose, dithiothreitol, urea and phenol were purchased from BRL or American Research Products Company (AMRESCO) (Solon, OH). Low melting point SeaPlaque was obtained from FMC Corporation (Rockland, ME).

SeaPlaque was obtained from FMC Corporation (Rockland, ME). Acrylamide, N,N'-methylene-bisacrylamide, TEMED, and ammonium persulfate were ordered from BIO-RAD (Richmond, CA) or AMRESCO. Acrylamide stock solution (40 %, w/v) (ACRYL-40) and N,N'-methylene-bis-acrylamide stock solution (2 %, w/v) (BIS-2) were obtained from AMRESCO. 5-bromo-4-cloro-3-indolyl-β-D-galactoside (X-gal) was ordered from United State Biochemical Co. (Cleveland, OH). Isopropyl-β-D-thiogalactopyranoside (IPTG), 2(morpholino)ethanesulfonic acid (MES), Tris-(hydroxymethyl)aminomethane (Tris), hexamine cobalt chloride, polyvinylpyrrolidone (PVP), thylenediaminetetra-acetic acid (EDTA), tetramethylammonium chloride, sodium dodecyl sulfate (SDS), polyethyleneglycol (PEG), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), uridine, 5,5'-dithiobis(2-nitrobenzoate) (DTNB), phenylmethylsulfonyl fluoride (PMSF), Cibacron Blue 3GA-Agarose (Type 3000-CL-L), Sephadex G-100, ampicillin, kanamycin, 3-(N-Morpholino)-propanesulfonic acid (MOPS), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitro blue tetrazolium (NBT) were purchased from Sigma. Rubidium chloride was ordered from Morton Thiokol. Inc. (Danvers, MA). Premade Sephadex G-50 (Select-D) columns were from 5 Prime - 3 Prime, Inc. (Paoli, PA). Nitrocellulose filters were ordered from Schleicher and Schuell (Keene, NH). Films (XAR-5 and XRP-5) were from Eastman Kodak Co. (Rochester, NY). Polaroid films were purchased from Polaroid Co. (Cambridge, MA).

2.1.3 Radio-isotope Labelled nucleotides and Oligonucleotides

Deoxynucleoside triphosphates labeled at the alpha position and ribonucleoside triphosphates labeled at the gamma position with ^{32}P as well as [α-^{35}S] dATP were products of ICN Biomedicals, Inc. (Irvine, CA) and New England Nuclear (NEN)/Du Pont Co. (Wilmington, DE). Oligonucleotides were synthesized by solid support method (Matteucci & Caruthers, 1981) in an automated DNA synthesizer (Applied Biosystems, Model 380A) and purified according to the catalog for "Oligonucleotide Purification Cartridges" and the protocal from Khorana (personal communication) (Appendix IV). The synthetic oligonucleotides used in this work are listed in Appendix III.

2.1.4 Bacterial Strains and Vectors

The bacterial strains used in this work as well as their genotypes and references are listed in Table 5.

Plasmid containing *Drosophila melanogaster adh* (psAC1) was a gift from Sofer. The expression vector with the lambda PL promoter (pPL2) and the plasmid with a cI857ts gene (pCI857) were gifts from Khorana. M13mp18 and M13mp19 were ordered from BRL.

Table 5.

Bacterial Strains

E. coli Strain	Genotype	Reference
BW313	HfrKL16 PO/45[*lysA*(61-61)], *dut1*, *ung1*, *thi1*, *relA1*.	Kunkel, 1985
CSH50	[Δ(*pro-lac*)], *ara*, *thi*, *strA/F'*, (*traD36*, *proAB*, *lacI$_q$Z*$^-$ M15).	Kunkel 1985
JM110	*(pro-lac)*, *dam*, *dcm*, *rpsL*, *thr*, *leu*, *thi*, *lacY*, *galK*, *galT*, *ara tonA*, *tsx*, *supE44*, (F', *traD36*, *proAB*, *lacI$_q$Z*$^-$ M15)	Yanisch-Perron, *et al.*, 1985
DH5αF'	F', *endA1*, *hsd R17*, *supE44*, *thi*, *recA1*, *gryA96*, *relA1*, λ$^-$, 80d*lacZ* M15, (*lacZYAargF*)U169	BRL Cat. No. 8264SA
M5219	N$^+$, F$^-$*lacZ*, *trp*, *Sm^R*, *gal3*, *chl*, *bio*, *uvrB*, (λ*N7N53cI857*H1)	Patrick, *et al.*, 1981
LC137	*htpR^{165}*, *lon^{R9}*, *lac*, *trp*, *pho*, *rpsL*, *supC^{ts}*, *mal*, *tsx::Tn10*	Goff *et al.* 1984

2.2 Methods

The details of experimental procedure and common reagents used in the following methods are described in Appendices IV and V.

2.2.1 Construction of the Full-length *Adh* cDNA by Site-directed Deletions - Kunkel's Method

In order to express *Drosophila melanogaster* alcohol dehydrogenase (DmADH) in *E. coli*, it was necessary to construct a DmADH cDNA. As mentioned in Chapter One, the DmADH coding region contains two small introns (65 bp and 70 bp). Deletion of the two introns in DmADH by Kunkel's method of site-directed mutagenesis (1985) (Appendix IV) is an efficient way to achieve the purpose. A 3.2 kilobase *Xba*I fragment containing Dm*Adh* was excised from psAC1 and subcloned in M13mp18. Single-stranded M13/*Adh* containing uracil was prepared in an *E. coli* host defective in dUTPase and uracil glycosylase (BW313) as described by Kunkel (1985). This single-stranded template was hybridized with synthetic primer (INTRON1 or INTRON2) containing the base sequence corresponding to seven codons in the carboxyl end of one exon and seven codons in the amino terminus of the adjacent downstream exon (Fig. 4). The complementary strand lacking uracil was synthesized *in vitro* along the M13/*Adh* template using DNA polymerase I (Klenow) in the presence of all four deoxynucleoside triphosphates.

Transformation of a dut$^-$, ung$^-$ *E. coli* host (CSH50) with this heteroduplex DNA selects against the uracil-containing strand, so that progeny phage should contain predominantly Dm*Adh* without introns. This prediction was confirmed by DNA sequence analysis.

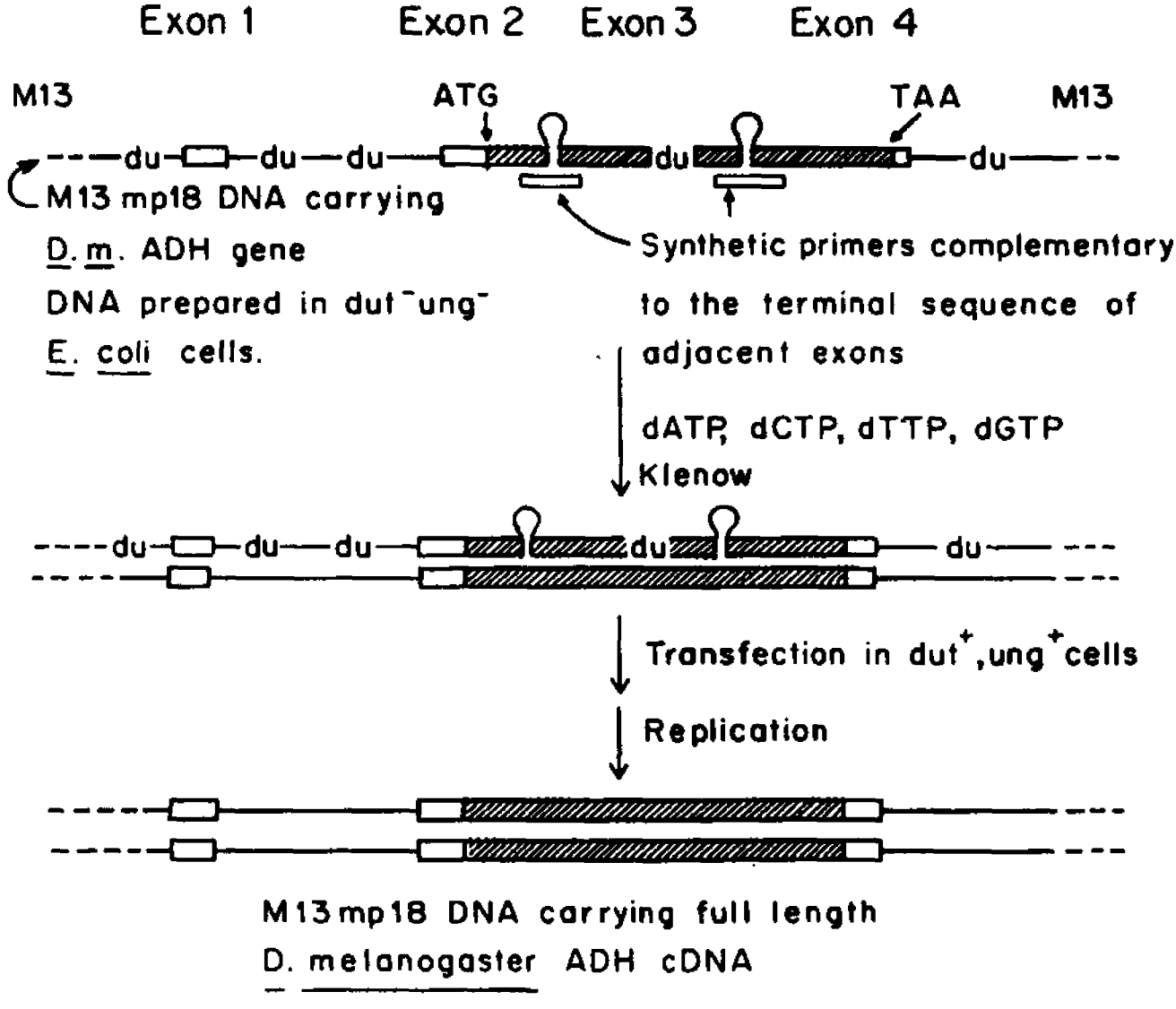


Fig. 4 **Construction of DmADH cDNA by Kunkel's method.** The method is described in Section 2.2.1 and Appendix Four. The uracil containing template was hybridized with synthetic mutagenic primers. After a complementary strand was synthesized *in vitro*, the double strand M13 was transformed into *E. coli* CSH50.

2.2.2 Subcloning and Expression of DmAdh cDNA

An M5219/pPL2 system is used for Dm*Adh* expression. The expression vector pPL2 contains a lambda PL promotor which is highly efficient for transcription and can be controlled by lambda repressor (lambda cI gene product). *E. coli* strain M5219 carries a defective prophage with cI857 thermo-sensitive mutation, thus providing a convenient control for lambda PL promotor.

Subcloning of *Adh* cDNA into pPL2 is shown in Figure 5. One modification to M13/*Adh* cDNA was made in order to facilitate the subcloning. An *Eco*RI site was introduced by Kunkel's method of site-directed mutagenesis immediately upstream of the ATG start codon. Since there is a unique *Bal*I site 244 bp downstream from the TAA termination codon, the new *Eco*RI site enabled us to excise the entire DmADH coding sequence. After mung bean nuclease digestion which creates a blunt end from the cohesive termini produced by *Eco*RI digestion, the *Adh* cDNA was inserted into pPL2 at 12 bp downstream from the Shine-Dalgarno sequence (Guarente *et al*, 1980). This construct preserves the correct translation reading frame.

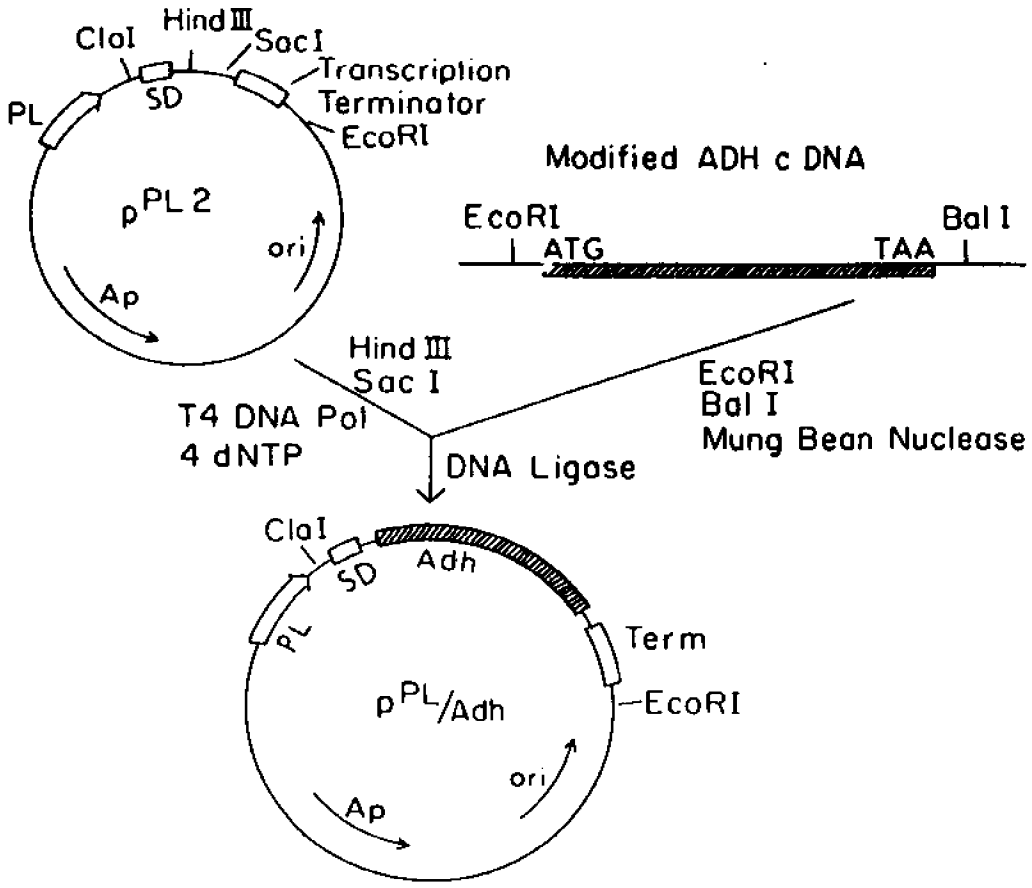
pPL2 was digested with *Hin*dIII and *Sac*I and purified by gel electroelution. The 5'-protruding terminus generated by *Hin*dIII was filled by the polymerase activity of T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates. The 3' overhang of the *Sac*I site was removed by the 3' to 5' exonuclease activity of the same enzyme. The

vector and *Adh* cDNA were joined by T4 DNA ligase. This plasmid construct (pPL/*Adh*) codes for wild-type ADH.

To produce point mutation, a wild-type *Adh* cDNA was excised from pPL2/*Adh* with *Cla*I and *Eco*RI. The *Adh* insert fragment was cloned into M13mp19 and digested with *Eco*RI and *Acc*I. The *Acc*I site then was mutated to *Cla*I, making the *Adh* insert fragment easy to subclone back and forth between pPL2 and M13 vectors.

E. coli M5219 was transformed with pPL2/*Adh*. Colonies grown in the presence of ampicillin were screened for *Adh* by colony hybridization (Maniatis *et al.*, 1982). Liquid cultures of positive colonies were grown to mid-log phase at 28°C, heat shocked at 42°C for 30 min, and incubated at 37°C for three hours. Cells from the cultures were harvested and sonicated. DmADH was identified by an enzyme activity assay (Lee, 1982) (Appendix IV) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results of SDS-PAGE were analyzed by densitometer scanning (Bio-Rad, Model VD-620).

In order to express protease-sensitive mutants of DmADH, protease defective *E. coli* LC137 was co-transformed with the pPL2/*Adh* and plasmid pCI857. Colonies were grown in the presence of ampicillin and kanamycin. Liquid cultures of transformants were grown to mid-log phase at 30°C, heat shocked at 42°C for 15 min, and incubated at 37°C for 4 hours.



Transformation of E. coli M 5219
28°C until mid log phase
42°C, 30 min; 37°C, 3hr.
Harvest cells
Purify Drosophila ADH

Fig. 5 **Subcloning of DmADH cDNA into pPL2.** The experimental procedures have been described in Section 2.2.2. *Adh* cDNA digested by *Eco*RI and *Bal*I was subcloned at the *Hin*dIII and *Sac*I sites by blunt end ligation. The recombinant plasmid was used to transform *E. coli* strain M5219. *Adh* cDNA was then excised from pPL2/*Adh* with *Cla*I and *Eco*RI and cloned into M13mp19 for further purposes.

2.2.3 Purification of DmADH from *E. coli*

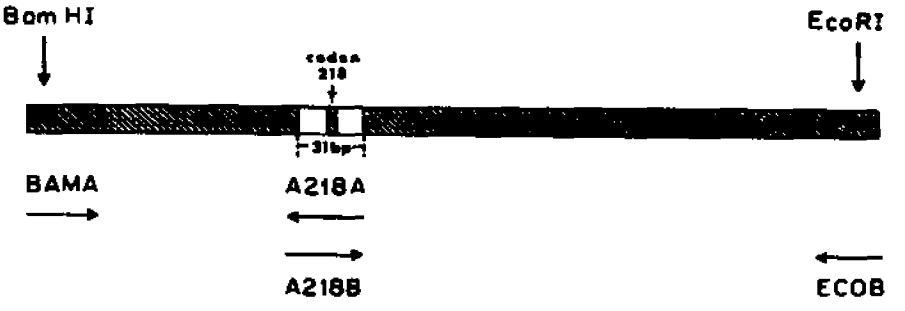
Large scale cultures of transformed cells at late log-phase were pelleted at 4°C, 7,000 x g for 30 min and resuspended at 10% by weight in sonication buffer (Appendix V). Cell suspensions were sonicated for 5 min on ice (Heat Systems, Model W-225R), and cell debris was removed by centrifugation at 23,000 x g. The supernatant was adjusted to 40% ammonium sulfate saturation and centrifuged to remove precipitated proteins. Ammonium sulfate concentration was increased to 60% saturation; the resultant protein precipitate was dissolved in column buffer (Appendix V) and applied to a Sephadex G-100 column (2.5 x 120 cm). Eluted fractions containing the majority of ADH activity were pooled and applied to a Cibacron Blue 3GA-Agarose column (2.5 x 20 cm) equilibrated with the same column buffer. Proteins were eluted by a NaCl gradient (0 to 2 M). DmADH was eluted at approximately 1.5 M NaCl, concentrated in an Amicon ultrafiltration cell, and stored at -20°C. Aliquots from each step of purification were analyzed for protein concentration and ADH activity.

2.2.4 Creating Point Mutations in DmADH

Five mutations were created in these studies: GA14 (Gly-14 to Ala), GV14 (Gly-14 to Val), CA135 (Cys-135 to Ala), CA218 (Cys-218 to Ala), and CA135/CA218 (Ala substitutions at both Cys-135 and Cys-218). Mutagenesis for the first three was carried out by Kunkel's method (Appendix IV).

Several attempts to construct the CA218 mutant using Kunkel's method were unsuccessful. We then used polymerase chain reaction (PCR) (Higuchi *et al.*, 1988) (Appendix IV) to generate the CA218 mutation. Four synthetic primers were prepared to produce a 655 bp partial DmADH cDNA fragment which has Cys to Ala mutation at position 218, a *Bam*HI site at the 5' end and an *Eco*RI site at 3' end (Fig. 6). A218A and A218B are mismatched primers to make the mutation at position 218; BAMA and ECOB are the primers containing respectively *Bam*HI and *Eco*RI linkers. The first step in this method was to make a 270 bp DNA fragment primed by A218A and BAMA, and a 416 bp DNA fragment primed by A218B and ECOB using PCR conditions described by Higuchi *et al.*(1988) and the protocol for "GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus) (Appendix IV). These two fragments are complementary for 31 bp centered at the locus of CA218 mutation. A mixture of 0.3 pmole of each fragment produced from the step one was extended for 20 cycles of PCR in the presence of 50 pmole each of BAMA and ECOB. The 655 bp DNA fragment produced was linked to pPL2 vector containing wild-type DmADH cDNA at the *Bam*HI and *Eco*RI sites by DNA ligase. This procedure constructed a pPL2 derivative containing the CA218 mutation. To generate the CA135/CA218 double mutant, the 655 bp PCR fragment was inserted in the same *Eco*RI and *Bam*HI sites of the CA135 cDNA.

A.



B.

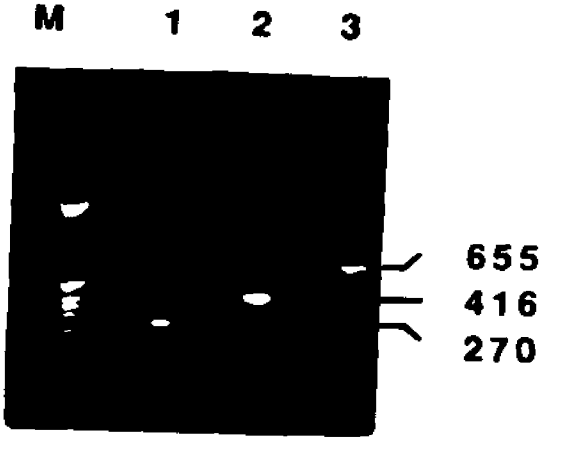


Fig. 6 A. **Diagram of partial *Drosophila* ADH cDNA and the primers used for PCR.** Total length of this region is 655 bp. B. **Agarose gel electrophoresis showing the three fragments generated by PCR.**

2.2.5 Western Blot of DmADH

Identification of cloned proteins on SDS-PAGE using an antibody against DmADH was described in Batzer *et al.*, (1988) (Appendix IV). Samples for SDS-PAGE were prepared from crude extracts of *E. coli* M5219 transformed with the expression vector pPL2 containing or lacking *Adh* cDNA.

2.2.6 Determination of Kinetic Parameters and Inhibition Studies

ADH activity was determined spectrophotometrically at 340 nm in the presence of two substrates, alcohol (2-propanol or ethanol) and NAD, in 100 mM Tris-Cl buffer (pH 8.7 or specialized) at 25°C. Figure 7 shows the time course of absorbance at 340 nm recorded by a spectrophotometer (Gilford-Response). The initial velocity (absorbance changes per min) can be calculated by the spectrophotometer. All Km and Vmax values were calculated from duplicate initial velocities determined at five substrate concentrations. Ki values were calculated from Km values determined as above at increasing concentration of the inhibitor (pyrazole: 0 µM, 5 µM, 10 µM; NADP: 0 µM, 100 µM and 500 µM). Km and Vmax were calculated by ENZFITTER program (Elsevier-BIOSOFT) on a personal computer. Reactions were initiated by adding ADH. Concentrations of NAD and alcohol were specified as follow: the $K_{m(app)NAD}$ was determined for NAD between 0.1 and 1 mM with a constant alcohol concentration of 10 mM, and the $K_{m(app)Alc}$ for alcohol at concentrations from 2 to 10 mM with a constant NAD

concentration of 1 mM. The Ki for pyrazole was assayed by varying the ethanol concentration from 2 mM to 10 mM at a constant NAD concentration, while the Ki for NADP was measured by varying NAD concentration from 0.1 mM to 1 mM at a constant alcohol concentration.

2.2.7 **Thermal Denaturation Study**

Crude extracts of *E. coli* LC137 containing wild-type or mutant *Adh* cDNA were incubated at 40°C for 60 min. Aliquots of 100 µl from each sample were taken every 6 min and assayed for ADH activity (Lee, 1982) (Appendix IV). All assays were performed in duplicate.

2.2.8 **DTNB Modification of Wild-type and Mutated DmADH**

DTNB can react with the ionized cysteinyl residue to produce 5-thio(2-nitrobenzoate) (TNB) which shows λ_{max} at 412 nm (Riddles, *et al.*, 1979).

DmADH (10 µM) was mixed with DTNB (100 µM) in 1 ml of 0.2 M Tris-Cl buffer (pH 8.0) at 25°C in the presence or absence of 1 mM NAD and 10 mM 2-propanol. The absorbance at 412 nm was recorded at 0.25 min intervals on a spectrophotometer (Gilford-Response II).

2.2.9 DTNB Inactivation of Wild-type and Mutated DmADH

DmADH (4 µg) was incubated in 200 µl of 100 µM DTNB and 0.2 M Tris-Cl buffer (pH 8.0) at 25°C for 10 min. Aliquots of 20 µl from each sample were taken every 3 min and assayed for ADH activity in duplicate.

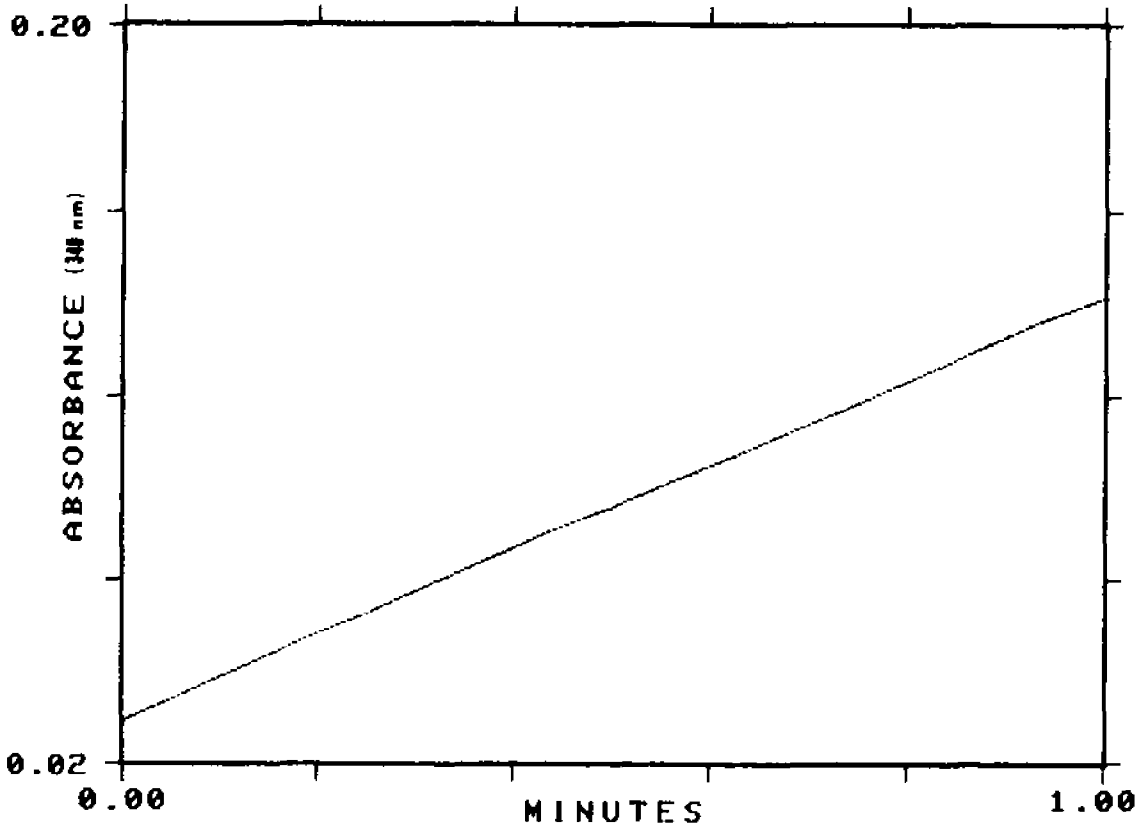


Fig. 7 **The time course of absorbance at 340 nm.** The velocity of DmADH catalysis is defined as the absorbance changes per min.

CHAPTER THREE

EXPRESSION OF WILD-TYPE AND MUTANTS *DROSOPHILA* ADH IN *E. COLI*

3.1 Construction and Expression of *Drosophila* ADH cDNA

Sequencing results show that the two introns of DmADH were deleted and that all three exons were fused correctly, preserving in-frame translation (Fig. 8) and that unintentional mutations had not been introduced.

SDS-PAGE (Fig. 10) shows that transformation of *E. coli* M5219 with pPL2/*Adh* provides a high level of protein expression and that DmADH produced in *E. coli* is the same size as the authentic protein (27 KDa). SDS-PAGE also shows that *Adh* inserted in the wrong orintation reveals a different restriction pattern (Fig. 9) and does not produce ADH.

3.2 Purification of DmADH from *E. coli*

The purification procedure has been described in Section 2.2.3. It includes ammonium sulfate precipitation steps, a gel filtration column (Sephadex G-100), and an affinity column (Cibacron Blue 3GA-Agarose). The results of wild-type DmADH purification are shown in Figure 11 and Table 6.

A maximum of 17-fold purification was achieved during the purification of wild-type DmADH from *E.coli* M5219. Based on SDS-PAGE, the DmADH has been purified to homogeneity. The limited purification is a result of the high expression of DmADH in the *E. coli* host.

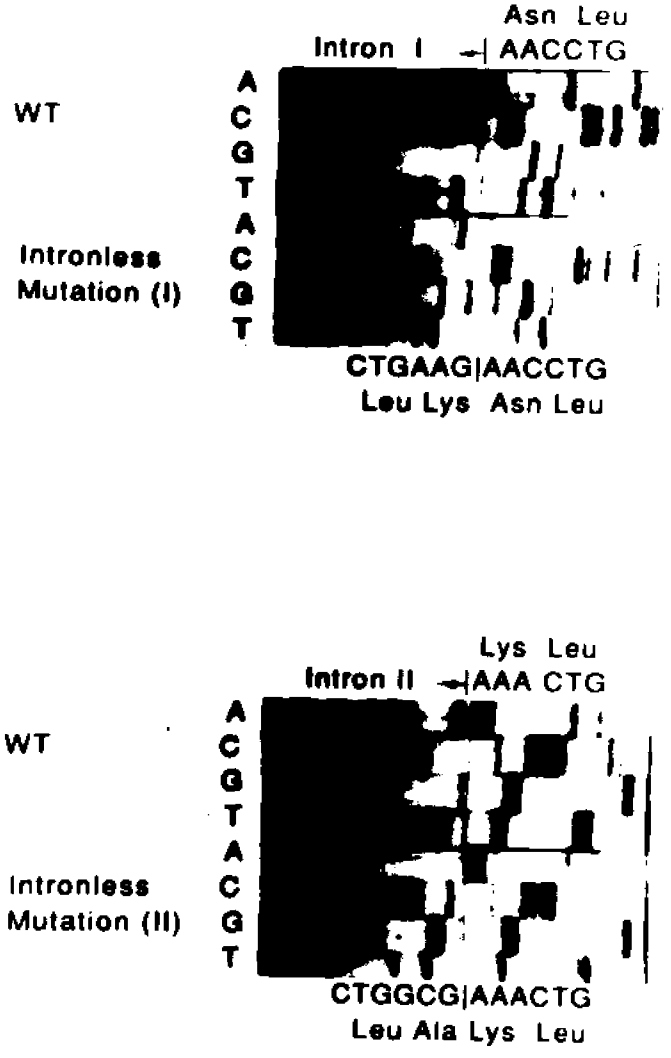
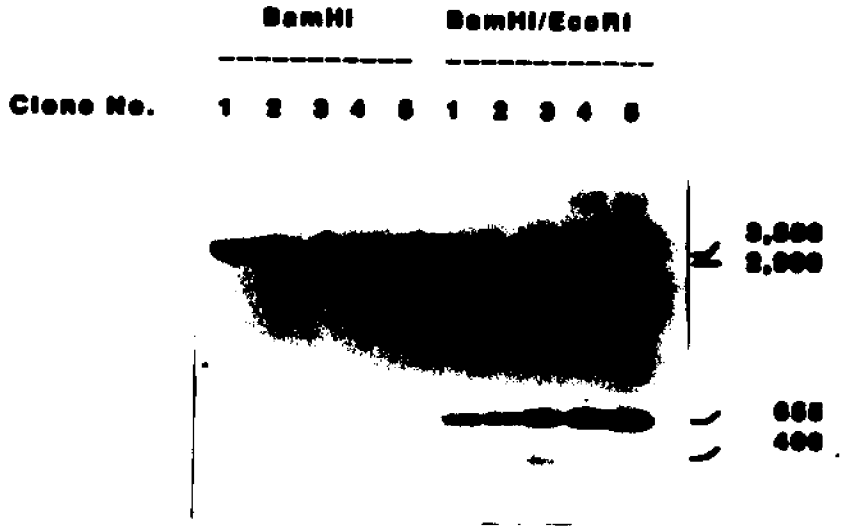


Fig. 8 **DNA sequence showing the two introns have been deleted.** The deletion was carried out by Kunkel's method (1985) (Section 2.2.1 and Appendix IV). Sequencing was performed by dideoxy chain termination method as described in Appendix IV.



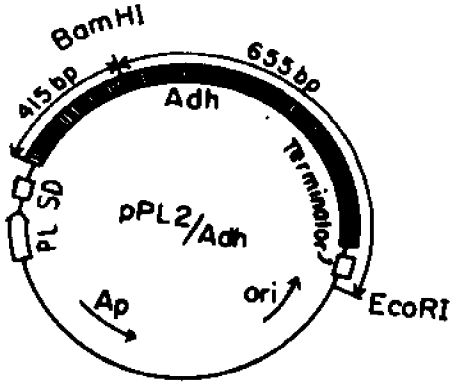


Fig. 9 **Southern blot showing the restriction pattern of *Adh* inserted in pPL2 expression vector.**

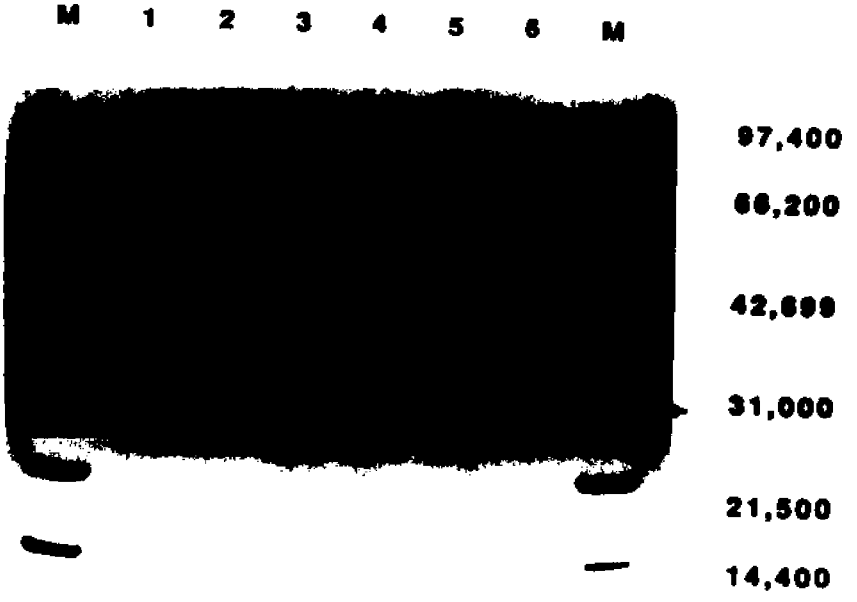


Fig. 10 **SDS-PAGE showing that pPL2/*Adh* produces ADH protein.** Samples were prepared from crude extract of *E. coli* M5219. Lane 1: M5219; Lane 2: M5219/pPL2; Lane 3: M5219/pPL2/*Adh* (the *Adh* clone showing a wrong restriction pattern which is clone 3 in Fig. 9); Lane 4: M5219/pPL2/*Adh* without heat shock; Lane 5: M5219/pPL2/*Adh* with heat shock; Lane 6: DmADH isolated from the fly; M = Size Marker (Bio-Rad).

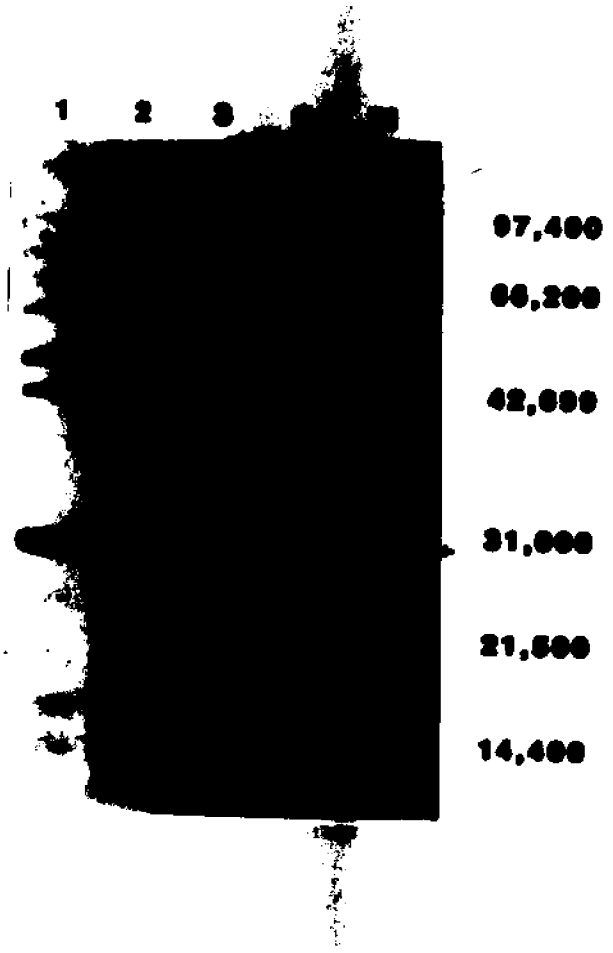


Fig. 11 **SDS-PAGE showing the purification of DmADH wild-type from *E. coli* M5219.** Lane 1: crude extract; Lane 2: after ammonium sulfate precipitation; Lane 3: after Sephadex G-100 column; and Lane 4: after Cibacron Blue column; Lane 5: M = Size Marker (Bio-Rad) .

Table 6.

Purification of DmADH from *E. coli* Host

Step	Volume ml	Protein mg	Total Activity 10^{-6} U*	Specific Activity 10^{-4} U*/mg	Fold of Purification
Crude Extract	50	688	3.6	0.52	1
Ammonium Sulfate	2	142	1.2	0.85	1.6
G-100 Sephadex	4	40.2	1.1	2.7	5.2
Cibacron Blue	3	6.0	0.54	9.0	17

* One unit of activity is defined as a change in absorbance at 340 nm of 0.001 per min (Sofer & Ursprung, 1968).

3.3 Mutations and Their Expression

In order to study the structure and function of DmADH, the following point mutations were introduced by Kunkel's and PCR methods: GA14 [Gly-14 (GGT) to Ala (GCT)], GV14 [Gly-14 to Val (GTT)], CA135 [Cys-135 (TGC) to Ala (GCC)], and CA218 [Cys-218 (TGC) to Ala (GCT)]. Figs. 12 and 13 are the sequencing results for these mutations.

There were no difficulties in expressing CA135 and CA218 in *E. coli* M5219. However, expression of GV14 is less than 10% of wild-type and GA14 expression is 70% of wild-type. Western blot analysis (Fig. 14) shows that *E. coli* M5219 produces ADH when transformed with either pPL2/*Adh* wild-type cDNA or mutant cDNA.

Expression of GV14 can be improved significantly in a protease defective host (LC137) (Goff *et al.*, 1984). Crude extracts of LC137 transformed by pPL2/*Adh* wild-type, GA14, and GV14 cDNAs were separated by SDS-PAGE (Fig. 15) and analyzed by densitometer scanning. The results show that GV14 protein expression is 66% of wild-type. However, the specific activity of ADH in the crude extracts of GV14 transformed *E. coli* LC137 is less than one percent of wild type.

Fig. 12 **DNA sequence showing the mutation of Gly-14 to Ala (GA14) and Val (GV14) in DmADH gene.** The mutagenesis was performed by Kunkel's method (1985) (Section 2.2.4 and Appendix IV). Sequencing was carried out by dideoxy chain termination method as described under the Appendix IV.

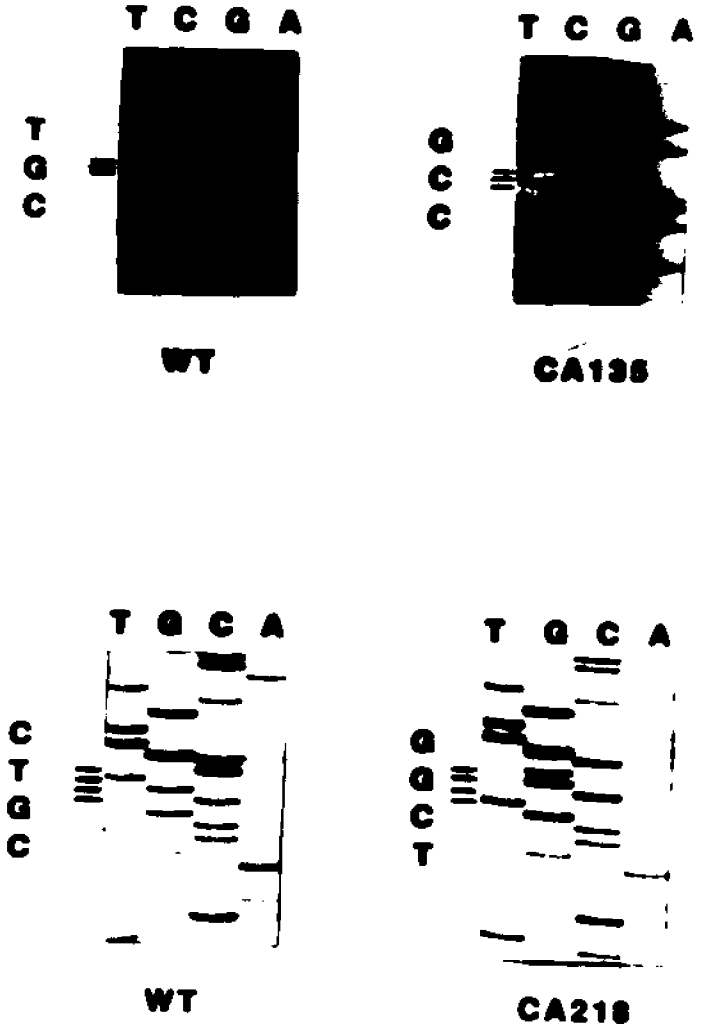


Fig. 13 **DNA sequence showing the mutation of Cys-135 to Ala (CA135) and Cys-218 to Ala (CA218) in DmADH.** The mutagenesis was carried out by both Kunkel's method (1985) and PCR (Higuchi *et al.*, 1988) (Section 2.2.4 and Appendix IV). Sequencing was performed as the same as in Fig. 12.

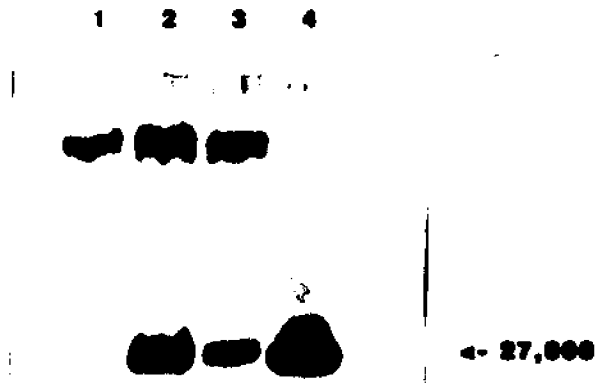


Fig. 14 **Western blot of wild-type and mutated DmADH proteins.** Crude extracts of *E. coli* M5219 transformed by the *Adh* cDNA plasmids were analyzed directly on SDS-PAGE. Immunochemical identification of cloned DmADH on SDS-PAGE was described in Batzer *et al.* (1988). Lane 1: pPL2, no *Adh* insert; lane 2: pPL2/GA14; lane 3: pPL2/GV14; Lane 4: purified wild-type DmADH.

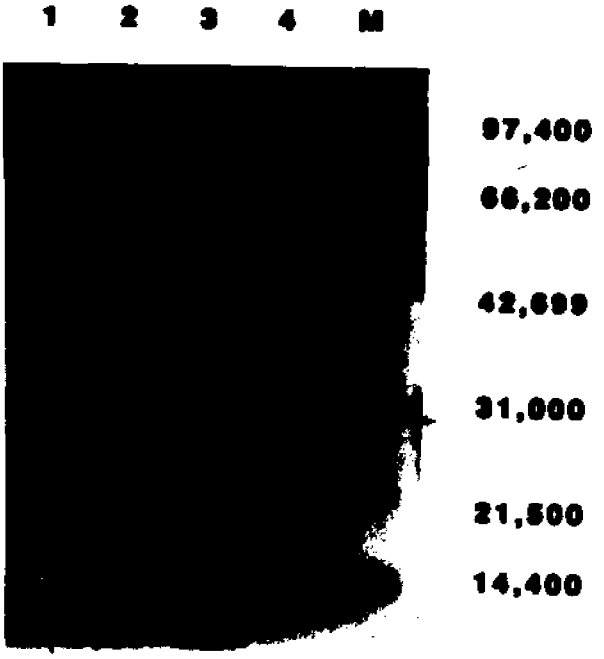


Fig. 15 **SDS-PAGE showing the expression of DmADH wild-type and mutants in E.coli LC137.** The samples were prepared directly from crude extracts of *E.coli* cells. Lane 1: LC137/pPL2/*Adh*; Lane 2: LC137/GA14; Lane 3: LC137/GV14; Lane 4: purified DmADH; M = Size Marker (Bio-Rad). Protein bands were visualized by Coomassie-blue staining (Bio-Rad).

3.4 **Discussion**

Site-directed intron deletion offers alternative way to construct cDNAs for eukaryotic genes. The traditional procedure for synthesis cDNA is by reverse-transcription. There are two major disadvantages to this method. First, the abundance of mRNA for single copy genes are usually low; secondly, reverse transcription terminates prematurely at a high frequency; thus, it is not easy to obtain a full length cDNAs from reverse transcription. Site-directed intron deletion, in contrast, is not suffered from these limitations. This method is suitable for a short gene with small numbers of intron. Introns up to 309 bp in length have been deleted by this method (Larson *et al.*, 1983). However, if there are large numbers of introns, it will be tedious to produce cDNA by site-directed intron deletion. It would also be impractical to delete introns which are several Kbp in length.

We achieved an overall 30% or greater mutation efficiency using the method developed by Kunkel (1985) and 14% by PCR (Higuchi *et al.*, 1988). In most cases, Kunkel's method is an efficient way to generate mutations. However, this method occasionally is unsuccessful as described in Section 2.2.4. It has been noticed that the mismatch repair system of *E. coli* can correct preferentially the newly made strand of heteroduplex (Leatherbarrow & Fersht, 1986). PCR has an advantage over Kunkel's method at this matter, since the mutagenesis is completed *in vitro*. However, DNA synthesis is carried out at high temperature (about 70°C) by *Taq* DNA polymerase in PCR so

that a high frequency of unexpected mutations becomes a major disadvantage in PCR methods.

Lower expression of GV14 in M5219 is not the result of codon preference by *E. coli* since expression can be increased by use of LC137, a protease deficient host. The difficulty observed in expressing GV14 in M5219 may be the result of protease sensitivity induced by structural changes in the mutated protein.

CHAPTER FOUR

USING SITE-DIRECTED MUTAGENESIS TO INVESTIGATE THE STRUCTURAL FUNCTION OF GLY-14 IN DmADH

As discussed in the "Introduction" (Section 1.2.2), glycine-14 in DmADH is the first residue of a "conserved sequence" (GlyXGlyX,Gly) in an putative NAD binding site of DmADH (Benyajati *et al.*, 1981). It may be critical for maintaining the tight turn structure required for correct conformation of the NAD binding domain. In order to determine the exact function of Gly-14, we created Gly-14 to Ala (GA14) and Gly-14 to Val mutants (GV14) by site-directed mutagenesis to investigate systematically the effect of the side chain at position 14.

4.1 Thermal Denaturation Studies

Chambers *et al.* (1984) showed that DmADH can be denatured by heating at 40°C. We carried out thermal denaturation on wild-type as well as mutated DmADH. The results (Fig. 16) show that wild type loses 30% of its activity after one hour at 40°C, whereas GA14 loses 90% of its original activity under identical conditions. Since the host strain, *E. coli* LC137, used for this experiment is deficient in proteases, it is unlikely that thermal inactivation of GA14 is caused by protease cleavage, although we can not completely rule out this possibility. Detection of thermal denaturation for GV14 is

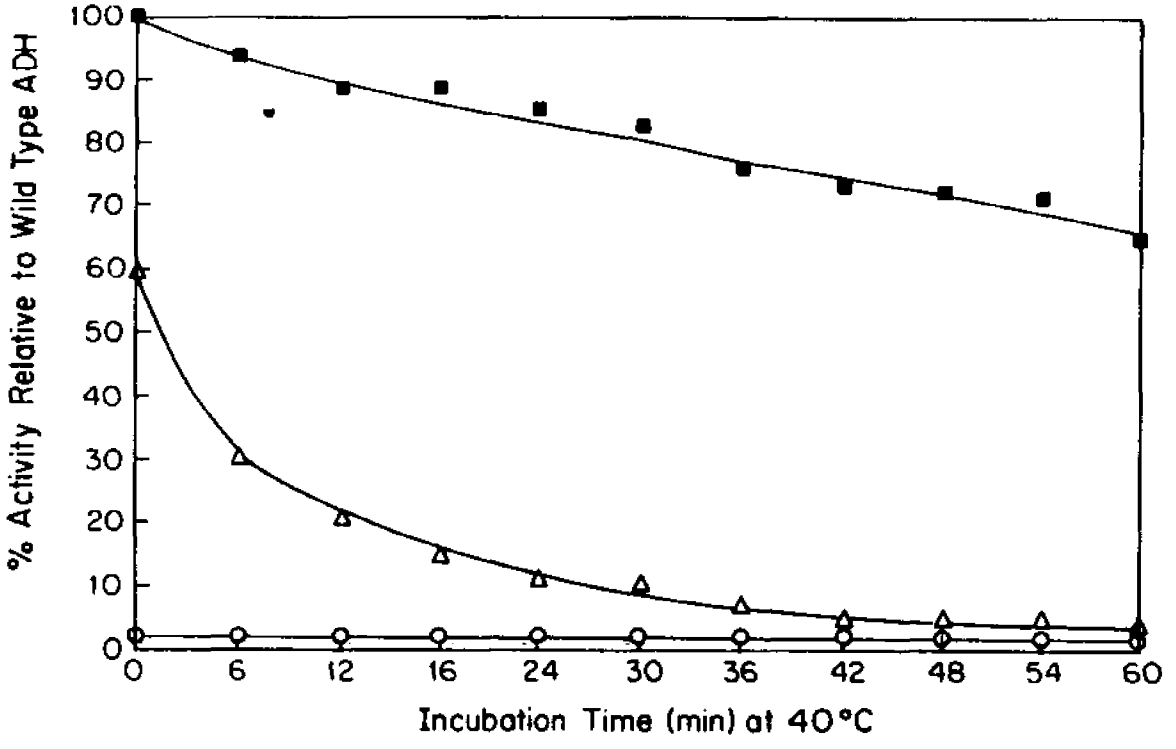


Fig. 16 **Thermal denaturation of wild-type and mutant DmADH.** Crude extracts (1 ml) of *E. coli* LC137 transformed with *Adh* DNA were incubated in sonication buffer, pH 7.4, at 40°C for 60 min. Aliquots of 100 µl were assayed for ADH activity every 6 min (Lee, 1982). Relative ADH activity is expressed as a percentage of wild-type initial velocity without heat treatment. ■ WT; △ GA14; and ○ GV14.

essentially impossible due to the extremely low specific activity of the mutated protein.

4.2 Kinetic Analysis of Wild-Type DmADH and GA14

We found that the kinetic behavior and substrate specificity of cloned DmADH is essentially identical to that of ADH isolated from the fruit fly (Table 7). Comparisons between cloned wild-type DmADH and its mutants can be extrapolated, therefore, to ADH isolated from *Drosophila*. The valine substituted mutant has almost no ADH activity, while the alanine substituted mutant shows decreased activity (69% of wild-type). Replacing Gly-14 with alanine increases the Km for NAD approximately three fold with little effect on Km for 2-propanol. Further, the $k_{cat}/K_{m(app)NAD}$ is decreased five fold in GA14, while the $k_{cat}/K_{m(app)Alc}$ shows no significant differences between wild-type and mutant. These results strongly suggest that the mutation in GA14 affects the binding of NAD but not of 2-propanol.

4.3 Inhibition Studies

Pyrazole is a competitive inhibitor of ethanol (Winberg *et al.*, 1982b). The inhibition constant for pyrazole is nearly identical in wild-type and GA14 (Table 8), suggesting that the alcohol binding domain is not affected by alanine substitution. On the other hand, we observed that NADP inhibits wild-type DmADH competitively while having almost no effect on GA14 activity (Fig. 17). These results indicate that the structure

of the NAD binding domain in GA14 has been changed such that NADP can no longer compete with NAD for binding to the active site.

Table 7.

Km(app) and k_{cat} Values for Cloned Wild-type and GA14[a]

ENZYME FORM	Km(app) mM		k_{cat} sec^{-1}	$k_{cat}/Km(app)$ $(sec)^{-1}(mM)^{-1}$	
	NAD	2-propanol		NAD	2-propanol
Wild-type (authentic)[b]	0.19	0.69			
Wild-type (recombinant)	0.12 ± 0.02	0.61 ± 0.09	2.8 ± 0.02	23.3	4.6
GA14	0.42 ± 0.03	0.56 ± 0.02	1.9 ± 0.01	4.5	3.4

[a] Enzyme were assayed as described under Section 2.2.6. The $Km_{(app)NAD}$ was determined for NAD concentrations between 0.1 and 1 mM with a constant alcohol concentration of 10 mM. The $Km_{(app)Alc}$ was measured for alcohol concentration from 2 to 10 mM with a constant NAD concentration of 1 mM.

[b] Data are from the reference (Chambers, 1984).

Table. 8

Ki Values of Pyrazole and NADP[a]

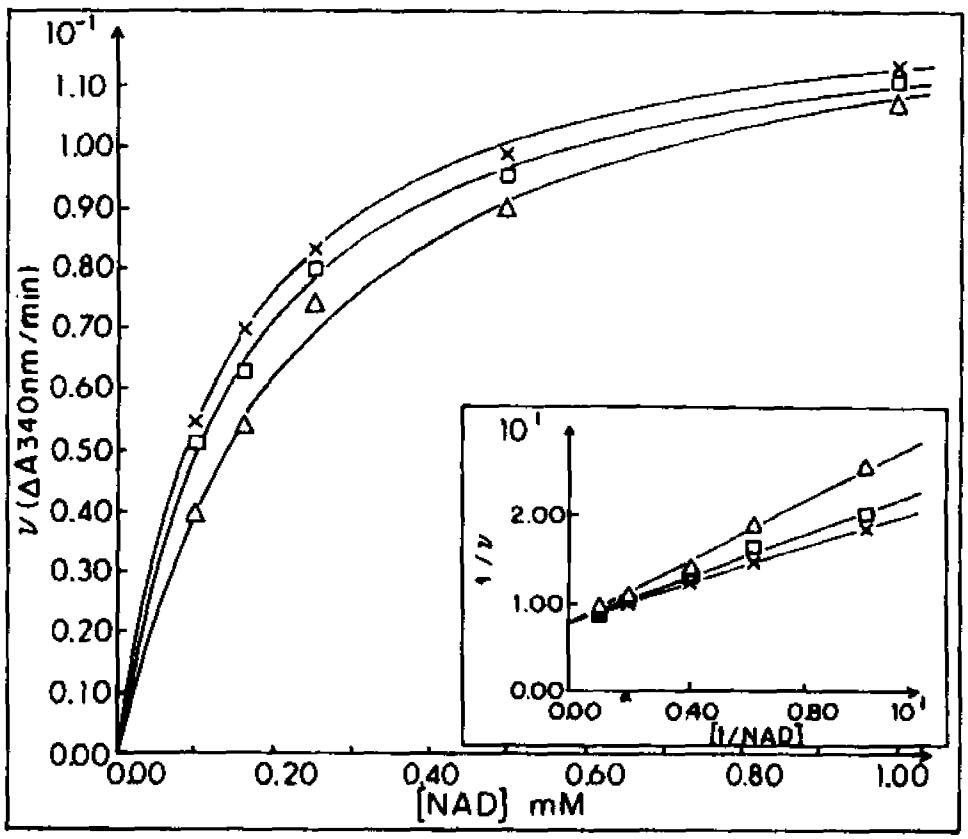
Inhibitor	Ki (μM)	
	Wild-type	GA14
Pyrazole[b]	3.49 ± 0.53	4.52 ± 0.02
NADP	716 ± 0.4	N.A.

[a]The inhibition constants (Ki) were obtained by ENZFITTER program as mentioned in Section 2.2.6. The Ki for pyrazole was assayed by varying the ethanol concentration, while the Ki for NADP was measured by varying the NAD concentration in 100 mM tris-Cl (pH 9.8).

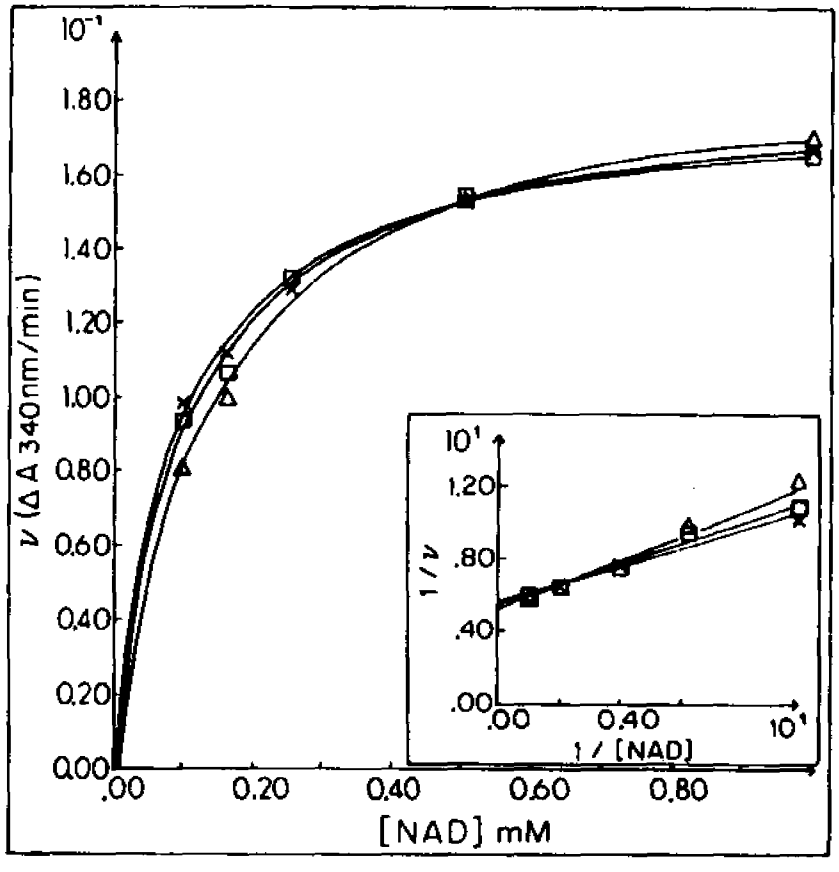
[b]The literature value of Ki for pyrazole is 4.4 μM (Winberg *et al.*, 1982).

Fig. 17 **Effect of NADP on wild-type and mutated DmADH activity.** These experiments were performed in 100 mM tris-Cl, pH 9.8, with a constant ethanol concentration of 10 mM at 25°C. Initial velocities were measured in the absence (X); and presence of (□) 100 μM; and (△) 500 μM NADP. A: wild-type DmADH; B: GA14.

A.



B.


10⁻¹
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
ν (Δ A 340nm/min)
.00
0.20
0.40
0.60
0.80
[NAD] mM
10¹
1.20
.80
.40
.00
1/ν
.00
0.40
10¹
1 / [NAD]

4.4 Discussion

DmADH differs from horse liver ADH in metal ion requirement, substrate preference, and stereospecificity of hydride transfer. When compared to ADH from other species, DmADH has little sequence homology, but the predicted secondary structure in the NAD binding domain resembles remarkably that of horse liver ADH (Benyajati *et al.*, 1981). Crystallographic studies of numerous NAD-dependent dehydrogenases reveal a common structure of six parallel β-sheets with five intervening α-helices (Rossmann, 1983). The first three β-sheets and two α-helices comprise the AMP binding unit (Rossmann *et al.*, 1975). A "conserved sequence" of $GlyXGlyX_2Gly$ is located at the sharp turn between the first β-sheet and α-helix in the nucleotide binding domain of many dehydrogenases (Argos & Leberman, 1985), kinases (Walker *et al.*, 1982), and oncoproteins (Sternberg & Taylor, 1984). Any disturbance of this tight turn diminishes nucleotide binding.

The glycine at position 14 of DmADH is the first residue in the "conserved sequence" and represents a logical site for studies on the role of this "conserved sequence" in the structure and function of NAD-dependent dehydrogenases, in general, and DmADH, in specific. We have systematically substituted glycine 14 in DmADH with amino acids having progressively larger side chains, i.e., alanine and valine. Our results show that GA14 is partially active while GV14 is almost inactive. GA14 is less stable than wild-type ADH at elevated temperature. These results are consistent with the

fact that an EMS-induced mutant, ADH^{n11}, which has a glycine to aspartate substitution at position 14, is inactive. Furthermore, our results prove that it is the size rather than the charge of the mutated residue which causes the inactivation of EMS-induced ADH^{n11}. Benyajati and her colleagues (1981) speculated that replacing glycine-14 with any larger residue would disturb the tight turn of the "conserved sequence", thus interfering with NAD binding. This view is supported by our results of comparing the kinetic parameters of GA14 to wild-type DmADH.

The k_{cat}/K_m is an apparent second-order rate constant that refers to the properties of the free enzyme and free substrate, and the activation energy of forming the enzyme-substrate complex (Fersht, 1985). The $k_{cat}/K_{m(app)}$ value for NAD in GA14 is five-fold lower than that of wild-type; however, the $k_{cat}/K_{m(app)}$ value for 2-propanol is essentially unaltered. These results indicate that the activation energy of enzyme-NAD binding is increased by alanine substitution; no effect on enzyme-alcohol binding is observed. Increased binding energy affects two factors: increased enzyme-NAD binding energy level, revealed by higher Km, and increased the activation energy for transition state, revealed by lower k_{cat}. Increased binding energy should result from structural changes in the NAD binding domain since no charged residue has been introduced. Because of the structural change, some weak bonds, such as hydrogen bonds, may be broken, thus disrupting the correct binding of NAD. Inhibition studies confirm this point. The Ki of

pyrazole for GA14 is essentially the same as that of wild-type, suggesting the structure of the alcohol binding domain is unchanged. In contrast, the inhibition caused by NADP is different for GA14 from that for wild-type. Noting that the only difference between NAD and NADP is an extra phosphate group on the ribose moiety of the adenine mononucleotide portion of the molecule, the failure of NADP to inhibit GA14 competitively indicates that NADP does not get into the NAD-binding domain of the mutated DmADH.

CHAPTER FIVE

TWO "ESSENTIAL" CYSTEINYL RESIDUES ARE NOT REQUIRED FOR THE CATALYTIC FUNCTION OF DmADH

It has long been thought that the two cysteinyl residues at position 135 and 218 in DmADH are required for catalytic function (Chambers, 1984). Chemical modification has been used to probe for the essential sulfhydryl group in this enzyme (Thatcher, 1981 and Chambers *et al.*, 1981) (Section 1.2.3). However, results have been ambiguous and contradictory, and the function of these sulfhydryl groups remains obscure.

In order to distinguish the reactive cysteinyl residue and investigate its functional significance, we have constructed a series of DmADH mutants containing alanine substitution at cysteinyl residues 135 (CA135), 218 (CA218), and both 135 and 218 (CA135/CA218).

5.1 Kinetic Parameters

Tables 9 and 10 show the kinetic parameters of purified mutants and wild-type DmADH with different substrates, 2-propanol and ethanol. Replacement of either cysteine 135 or 218 with alanine causes no decrease in ADH activity for 2-propanol or ethanol. Interestingly, the double mutant in which both cysteine residues are replaced by alanine (CA135/CA218) shows a 40% increase in activity over wild-type for 2-propanol. The single mutant in which the cysteine-135 residue is replace by alanine (CA135) shows 60% increase in activity over wild-

type for ethanol. In contrast, the replacement of cysteine 135, 218, or both results in unfavorable $K_{m(app)}$s for both alcohols. Specifically, CA218 causes a 2-fold increase $K_{m(app)}$ for 2-propanol and a 4-fold increase $K_{m(app)}$ for ethanol. However, these mutants have no effect on the $K_{m(app)}$ for NAD. The average results of a higher activity and lower binding affinity lead to insignificant changes of $k_{cat}/K_{m(app)}$ values except mutant CA135/CA218 (Tables 9 and 10). These studies suggest that the two cysteine residues are not directly involved in the catalytic function of DmADH, but serve as structural residues in the alcohol binding domain.

5.2 **DTNB Modification and Inactivation of DmADH**

Monitoring absorbance at 412 nm after DTNB treatment (Section 2.2.8) shows that the CA135 mutant has approximately the same sensitivity to DTNB modification as wild-type, but that CA218 is essentially insensitive to DTNB (Fig. 18). Calculations from the absorbance coefficient of TNB reveal that 1.2 moles of DTNB are converted into TNB per mole of DmADH monomer. This means that only one of the two cysteinyl residues in each monomer has been modified. This result is consistent with previous observations (Thatcher, 1981).

In the presence of DTNB, the wild-type enzyme and CA135, in which only residue 218 is cysteine, are inactivated rapidly; whereas CA218, in which only residue 135 is cysteine, and the double mutant are essentially unaffected (Fig. 19). These results clearly indicate that the cysteinyl residues at

Table 9.

$Km_{(app)}$ and k_{cat} Values of Wild-type and Mutant DmADHs with NAD and 2-propanol as Substrates[a]

ENZYME	Km mM		kcat sec^{-1}	kcat/Km $(mM)^{-1}(sec)^{-1}$	
	NAD	2-propanol		NAD	2-propanol
WT	0.12 ± 0.02	0.62 ± 0.09	2.90 ± 0.06	24	4.7
CA135	0.17 ± 0.01	1.17 ± 0.11	3.40 ± 0.09	20	2.9
CA218	0.15 ± 0.01	1.43 ± 0.09	3.12 ± 0.04	21	2.2
CA135/CA218	0.10 ± 0.01	1.11 ± 0.17	4.20 ± 0.08	42	3.8

[a]Enzymes were assayed as described under Section 2.2.6. The $Km_{(app)NAD}$ was determined for NAD concentration between 0.1 and 1 mM with a constant alcohol concentration of 10 mM. The $Km_{(app)Alc}$ was measures for alcohol concentrations from 2 to 10 mM with a constant NAD concentration of 1 mM.

Table 10.

$K_{m(app)}$ and k_{cat} Values of Wild-type and Mutant DmADHs with NAD and Ethanol as Substrates*

ENZYMES	Km mM		k_{cat} sec^{-1}	k_{cat}/Km $(mM)^{-1}(sec)^{-1}$	
	NAD	Ethanol		NAD	Ethanol
WT	0.12 ± 0.02	2.4 ± 0.4	1.4 ± 0.1	11.7	0.58
CA135	0.17 ± 0.01	3.1 ± 0.4	2.2 ± 0.1	12.9	0.71
CA218	0.15 ± 0.01	9.1 ± 0.6	1.1 ± 0.3	7.3	0.12
CA135/CA218	0.10 ± 0.01	6.3 ± 0.6	1.6 ± 0.4	16.0	0.25

* The enzyme assay was the same as in Table 9.

position 218 reacts with DTNB and this leads to inactivation of the enzyme. Since the results of site-directed mutagenesis shows that this cysteinyl residue is not required for DmADH activity, the DTNB-induced inactivation may result from a steric interference in the substrate binding domain.

5.3 **Substrate Protection Studies**

Figure 20 shows that DTNB modification of Cys-218 can be prevented by substrates, 2-propanol and NAD, indicating that Cys-218 is probably in or near the active site. This result is consistent with the significant changes with $K_{m(app)Alc}$ for mutant CA218 discussed in Section 5.1. On the other hand, Cys-135 which normally does not react with DTNB becomes sensitive in the presence of NAD and 2-propanol.

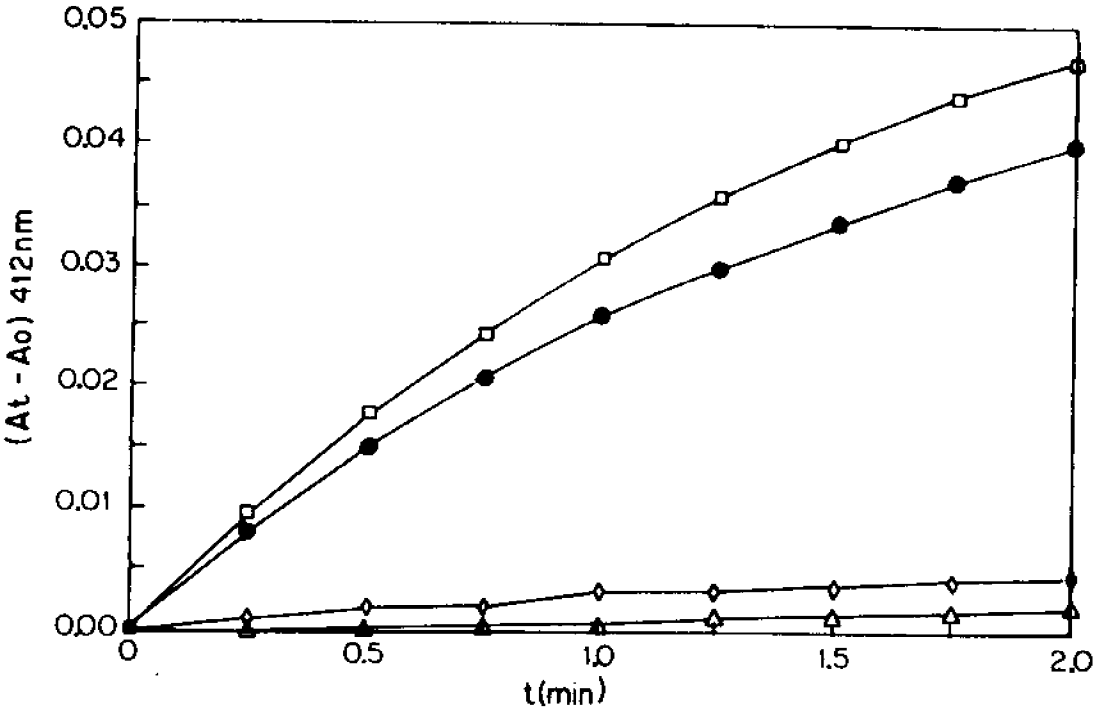


Fig. 18 **DTNB modification of wild-type and mutated DmADH.** Enzyme (10 nmole) was mixed with DTNB (100 nmole) in 1 ml of 0.2 M Tris-Cl buffer (pH 8.0) at 25°C. The absorbance at 412 nm was recorded. ●, wild-type; □, CA135; ◇, CA218; △, CA135/CA218.

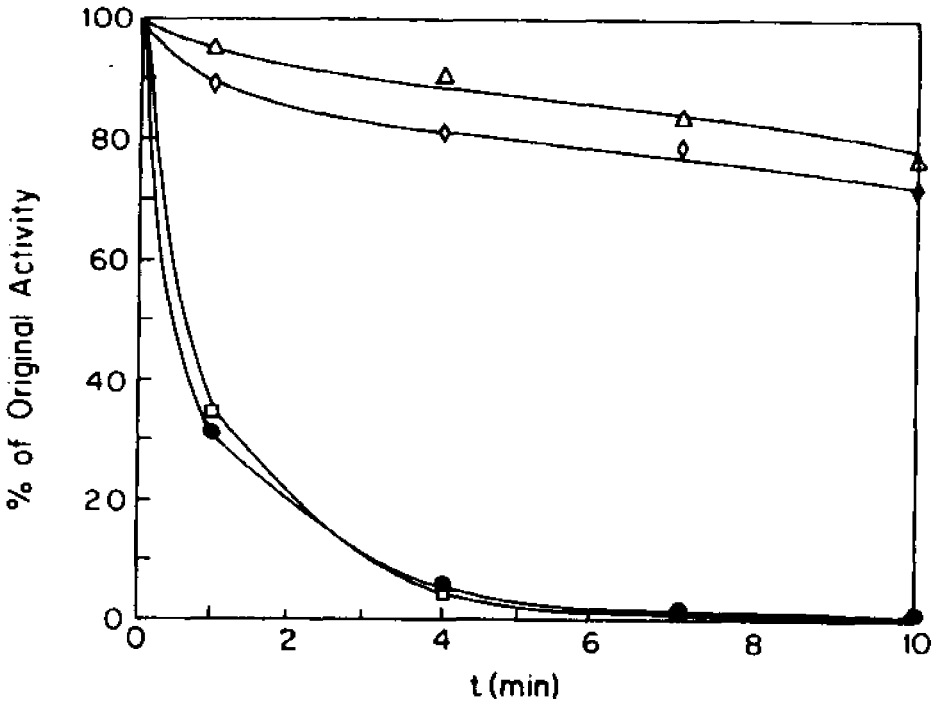
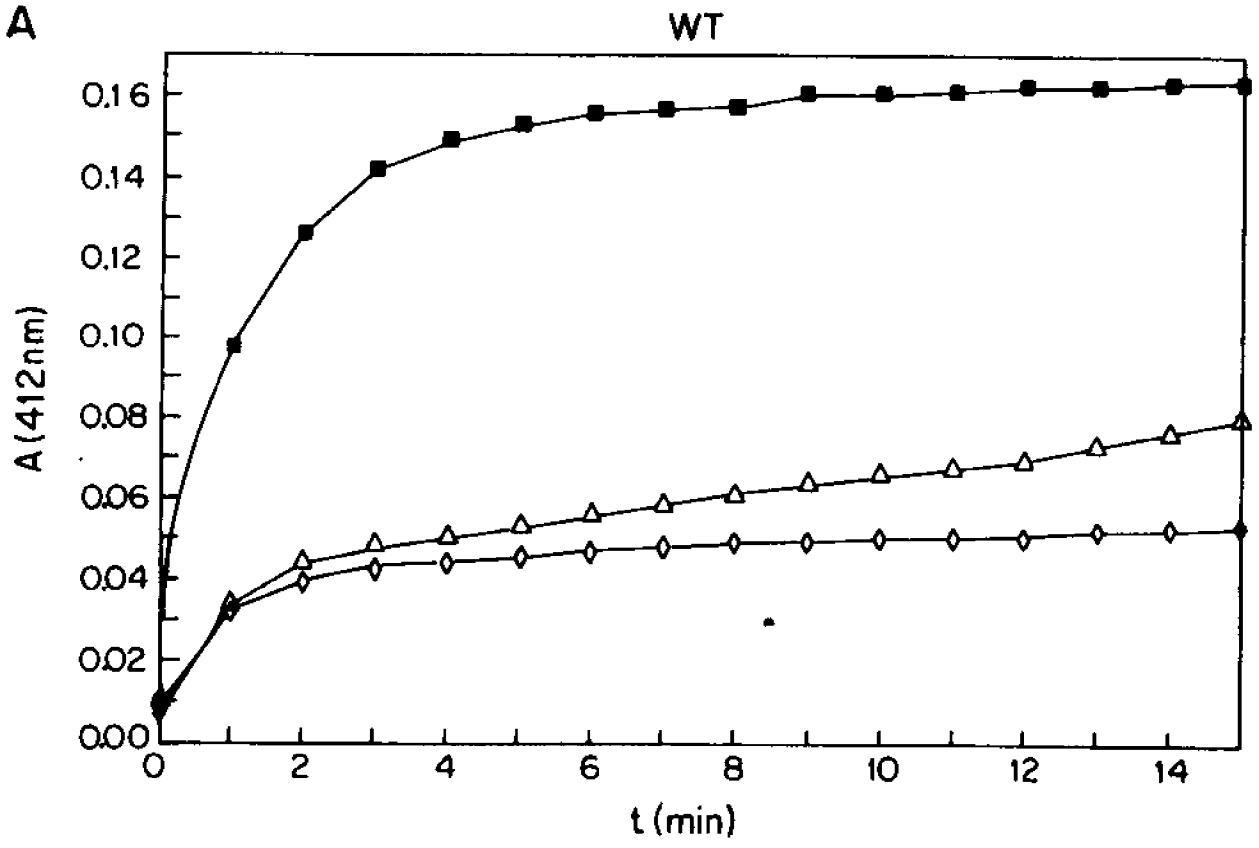
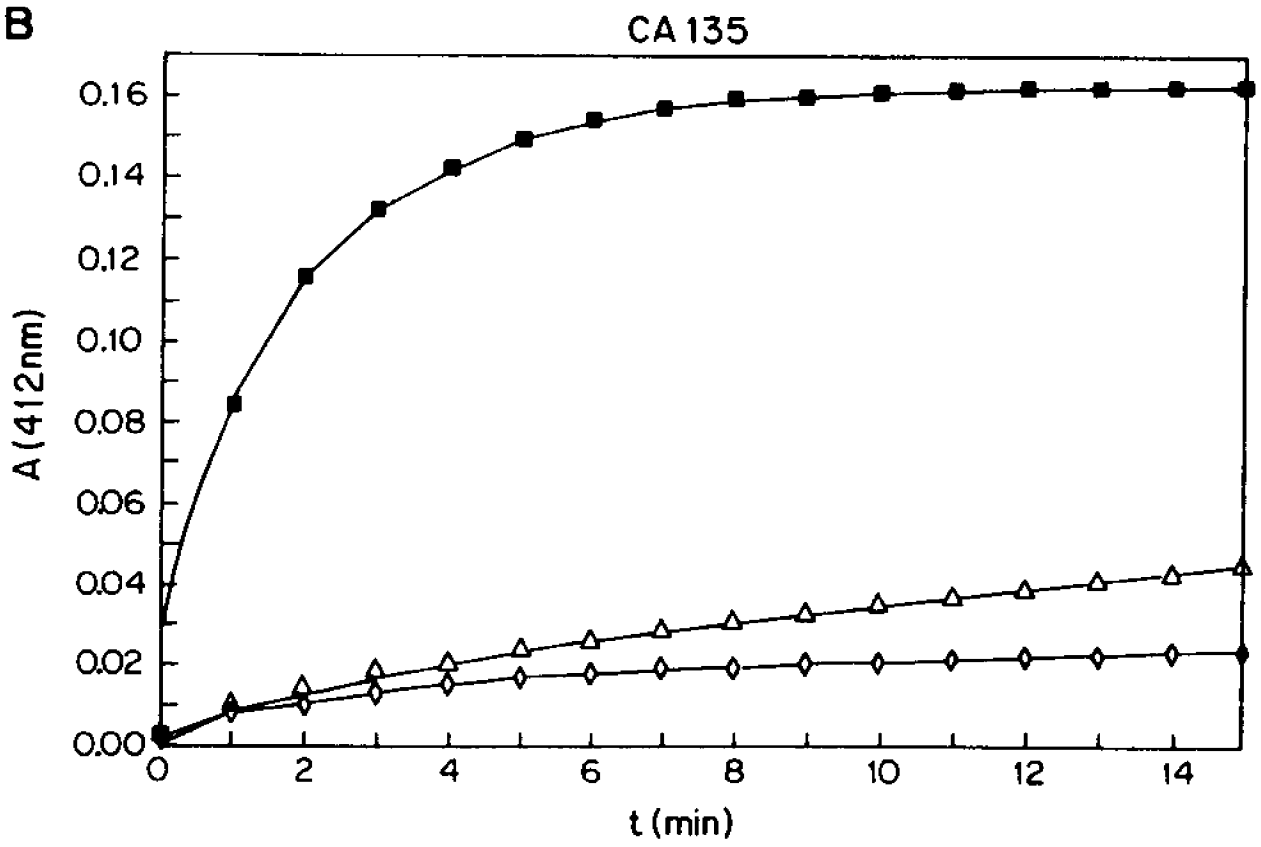
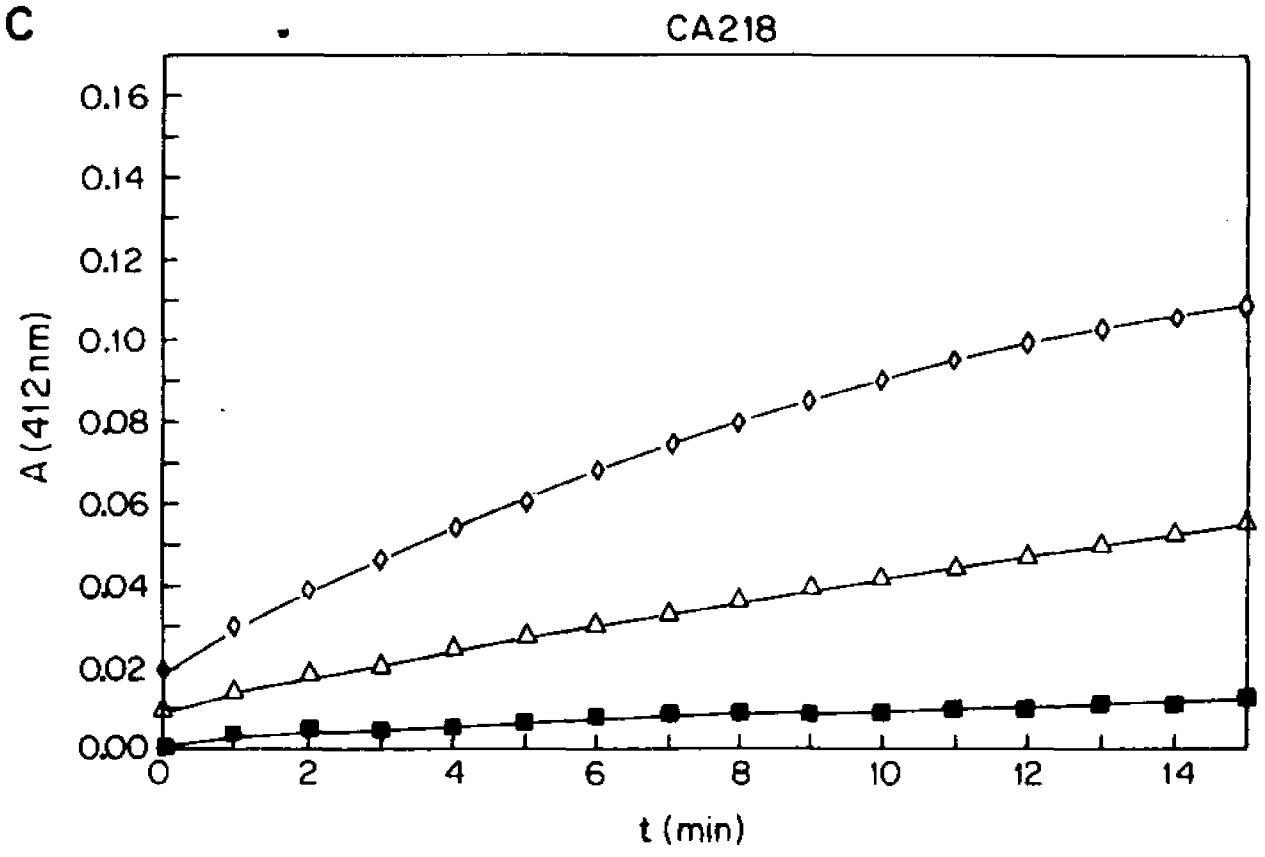


Fig. 19 **DTNB inactivation of wild-type and mutated DmADH.** The enzyme (4 µg) was incubated in 200 µl of 100 µM DTNB and 0.2 M tris-Cl buffer (pH 8.0) at 25°C. Aliquots of 20 µl from each sample were taken every 3 min and assayed for ADH activity. ● , wild-type; □ , CA135; ◇ , CA218; △ , CA135/CA218.

Fig. 20 **DTNB modification of wild-type and mutated DmADH in the presence and absence of NAD and 2-propanol.** Experimental conditions were the same as those described in Figure 17. A: wild-type; B: CA135; C: CA218; D: CA135/CA218. ■, absence of substrates; △, presence of 1 mM NAD; ◇, presence of 1 mM NAD and 10 mM 2-propanol.




B
CA 135
A (412nm)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
12
14
t (min)


C
CA218
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0.12
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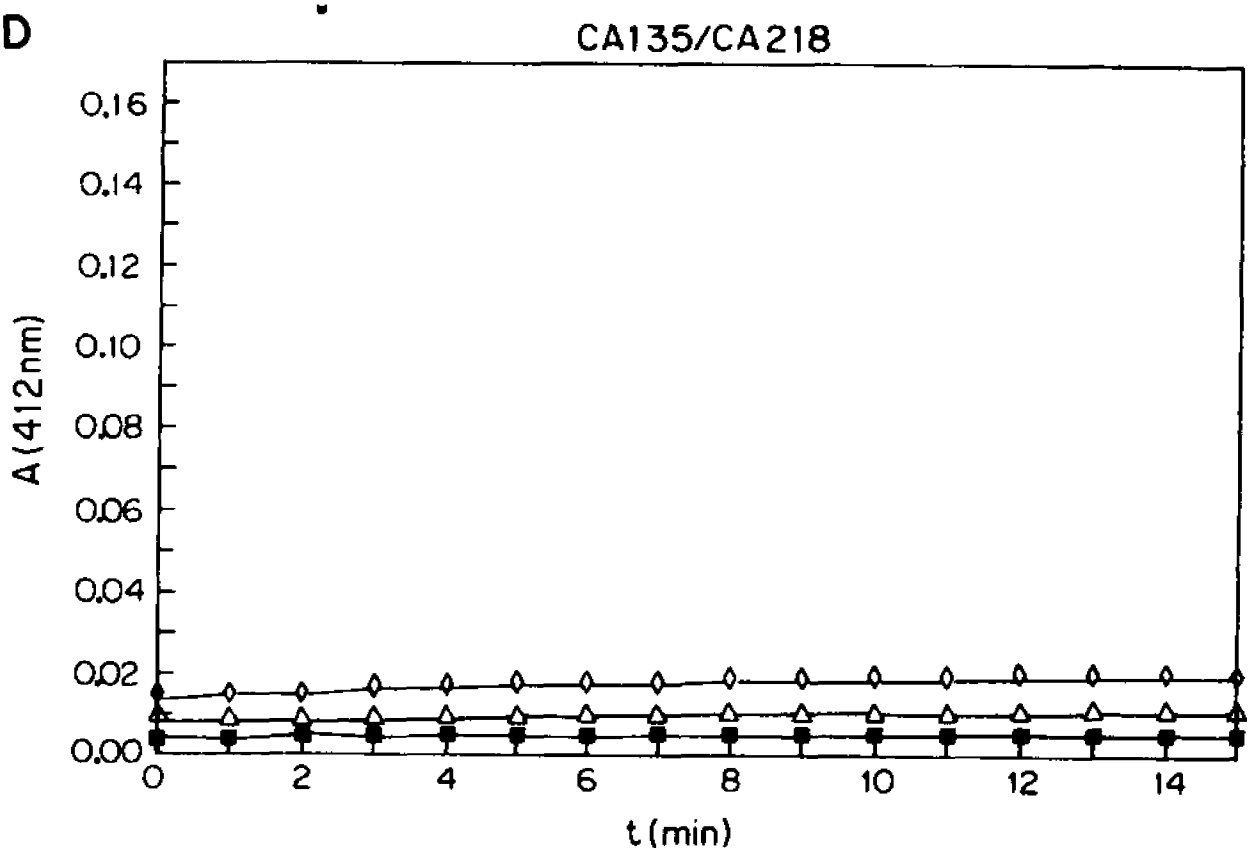
t (min)


D
CA135/CA218
A(412nm)
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0.14
0.12
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2
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8
10
12
14
t (min)

5.4 Discussion

In general, cysteinyl residues are considered potential catalytic sites in enzymes because of the nucleophilic nature of the sulfhydryl group. Previous studies on DmADH (Thatcher, 1981 and Chambers *et al.*, 1981) have found that this dimeric enzyme contains two cysteines per monomer, Cys-135 and Cys-218. In the native enzyme, one cysteine reacted rapidly toward DTNB with a concomitant loss of ADH activity. The second cysteine was almost unreactive toward DTNB in the native enzyme. However, the exact position and function of the reactive cysteinyl residue were not determined in these studies.

In order to investigate the role of these cysteines, we constructed mutants of DmADH in which the cysteinyl residues at position 135, 218 or both are replaced by alanine. All mutants show identical or elevated ADH activity relative to wild-type enzyme. These results strongly suggest that neither of the two cysteinyl residues is required for the catalytic activity of DmADH. To further clarify DTNB reactivity of these two cysteines, we treated wild-type DmADH and the three mutants, CA135, CA218 and CA135/CA218, with DTNB. The results of these studies show that the mutant CA218 is insensitive to DTNB modification and the treated protein remains enzymatically active while identical experiments carried out on CA135 show rapid DTNB reactivity with concomitant loss of ADH activity. A straight-forward interpretation of these observations is that Cys-218 is exposed to DTNB modification while Cys-135 is environmentally inaccessible to DTNB. These results are

different from previous observations (Chambers *et al.*, 1981) which suggested that Cys-135 was the DTNB-reactive residue and found that, after chemical modification by iodoacetamide, most of Cys-218 was recovered as unmodified cysteine. However, Cys-135 was never recovered from modified enzyme for conclusive identification because this residue is located in a large hydrophobic region. On the other hand, Thatcher (1981) indicated that DmADH was resistant to alkylation with iodoacetic acid or iodoacetamide. Therefore, chemical modification studies offer insufficient evidence to decide which cysteine is the reactive residue. In contrast, site-directed mutagenesis, coupled with chemical modification, provides an unambiguous identification of the DTNB accessible cysteine residue in DmADH.

Inactivation of an enzyme by a chemical reagent which modifies certain residues is often interpreted as evidence that the modified residue is critical for activity. This can be proven only by replacement of the suspect residue with other appropriate residues; for example, replacing cysteine by alanine or serine. In papain, the necessity of an active site sulfhydryl group has been strongly supported by the loss of activity upon replacement with glycine or serine (Clark & Lowe, 1978). In our case, replacing each cysteinyl residue with an uncharged alanyl residue does not alter the catalytic activity of this protein although chemical modification totally destroys the activity, suggesting that neither of the two cysteinyl residues in DmADH is required for catalytic function. Similar

to our observation, Profy and Schimmel (1986) reported that none of the three cysteinyl residues in the β-subunit of glycyl-tRNA synthetase was required for catalysis although enzyme activity was sensitive to N-ethylmaleimide (NEM) modification. They suggested that alkylation by NEM introduced a steric defect that led to inactivation of the enzyme. This probably is an appropriate explanation for our results.

The DTNB modification of Cys-218 can be prevented by the substrates, NAD and isopropanol, suggesting that Cys-218 may be in or near the active site. The kinetic studies show that mutant CA218 significantly alters binding affinity for both 2-propanol and ethanol with no effect on NAD binding. Therefore, we suggest that this cysteine in DmADH may play a structural or conformational role probably near the alcohol binding site rather than a catalytic role. To our surprise, Cys-135 becomes accessible to DTNB in the presence of NAD and 2-propanol. It is conceivable that these substrates induce a conformational change which is followed by the exposure of Cys-135 to DTNB modification.

CHAPTER SIX

SUMMARY

It is advantageous to construct an *E. coli* expression system to produce eukaryotic proteins, especially when the protein gene will be mutated several times and then expressed in varied forms. Generally speaking, an *E. coli* expression system includes a cDNA of the target protein, an expression vector with corresponding bacterial RNA polymerase recognition signals, and an *E. coli* host. This dissertation illustrates that site-directed deletion of introns in a small eukaryotic gene is an efficient way to obtain its cDNA. Further, M5219/pPL2 and LC137/pPL2/pCI857 are excellent expression systems for DmADH production.

By employing an *E. coli* expression system and site-directed mutagenesis, this dissertation first examines the structural role of glycine-14 in a "conserved sequence" which forms the tight turn located in the putative NAD binding domain. Mutating glycine-14 to valine virtually inactivates DmADH and alanine substitution causes a 31% decrease in activity, suggesting that glycine-14 is essential for DmADH activity. Thermal denaturation, kinetic, and inhibition studies confirm that replacing glycine-14 with either alanine or valine leads to structural changes in the NAD binding domain. This study provides direct evidence for the role played by glycine-14 in maintaining the tight turn structure. Replacement of glycine-14 with a residue having a bulky side

chain disrupts the tight turn formed by the "conserved sequence" GlyXGlyX$_2$Gly", thus interfering with the binding of NAD.

Second, this dissertation reports studies on the function of two cysteine residues, cysteine-135 and cysteine-218, which have been suggested to be catalytic residues in the enzyme. Substitution of one or both of these two cysteine residues by alanine does not affect the enzymatic activity of DmADH, indicating that neither of the cysteine residues is essential for catalysis. Chemical modification of wild-type and mutant proteins suggests that cysteine-218 may be in the vicinity of the active site and may play a structural role in the alcohol binding region.

In summary, site-directed mutagenesis of putative critical amino acid residues is an excellent tool for structural and functional studies of an interesting enzyme, such as DmADH. As mentioned in this dissertation, DmADH is unique among the ADHs and few of its structural and functional properties are known. Therefore, the results discussed in this dissertation about NAD binding and roles of the two cysteinyl residues should provide useful information about the structure-function relationships of DmADH.

ABBREVIATIONS

A	-	Adenine
ADH	-	Alcohol dehydrogenase
ATP	-	Adenosine triphosphate
BCIP	-	5-Bromo-4-chloro-3-indolyl phosphate
bp	-	Base pair(s)
BPB	-	Bromphenol blue
BSA	-	Bovine serum albumin
C	-	Cytosine
CA135	-	Cys-135 to Ala mutant
CA218	-	Cys-218 to Ala mutant
CA135/CA218	-	Cys-135 & Cys-218 to Ala double mutant
cDNA	-	Complementary DNA
DmADH	-	Drosophila melanogaster alcohol dehydrogenase
Dm*Adh*	-	DmADH gene
DMSO	-	Dimethylsulfoxide
DTNB	-	5,5'-dithiobis(2-nitrobenzoate)
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
EMS	-	Ethyl methanesulfonate
G	-	Guanine
GA14	-	Gly-14 to Ala mutant
GV14	-	Gly-14 to Val mutant
Hepes	-	N-2-Hydroxyethyl piperazine-N'-2-ethane sulfonic acid

IPTG	-	Isopropyl-β-D-thiogalac-topyranoside
kb	-	Kilobase pairs
k_{cat}	-	Turnover number in Michaelis-Menten model
kDa	-	Kilodaltons
Ki	-	Inhibition constant
$K_{m(app)Alc}$	-	Apparent Michaelis-Menten constant for alcohol
$K_{m(app)NAD}$	-	Apparent Michaelis-Menten constant for NAD
LADH	-	Liver alcohol dehydrogenase
MES	-	2-(morpholino)ethanesulfonic acid
min	-	Minute(s)
MOPS	-	3-(N-morpholino)propanesulfonic acid
NAD	-	Nicotinamide adenine dinucleotide
NADP	-	Nicotinamide adenine dinucleotide phosphate
NBT	-	Nitro blue tetrazolium
NEM	-	N-ethylmaleimide
nt	-	Nucleotide
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction
PEG	-	Polyethyleneglycol
PMSF	-	Phenylmethylsulfonyl fluoride
PFK	-	6-Phosphofructo-1-kinase
PVP	-	Polyvinylpyrrolidone
RF	-	Replication form
SDS	-	Sodium dodecyl sulfate
T	-	Thymine
TAE	-	Tris-Acetate EDTA buffer

TBE	-	Tris-Borate EDTA buffer
TE	-	Tris-EDTA buffer
TEMED	-	N,N,N',N'-tetramethylethylenediamine
TFB	-	Transformation buffer
TNB	-	5-thio(2-nitrobenzoate)
Tris	-	Tris(hydroxymethyl)aminomethane
Vm	-	Maximum velocity in Michaelis-Menten model
x-gal	-	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

Appendix I

AMINO ACID SEQUENCE OF DmADH

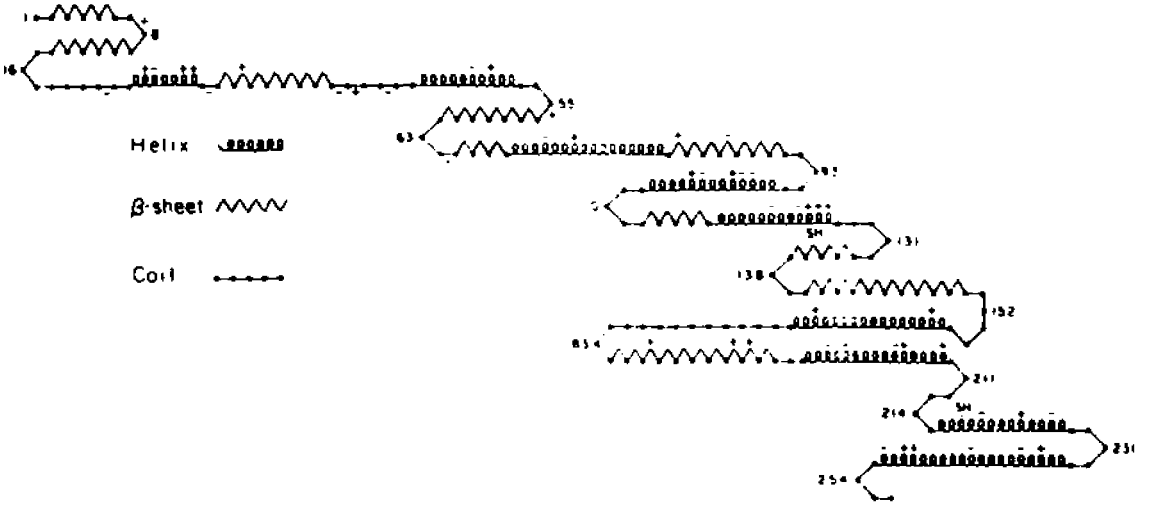
1 Ser-Phe-Thr-Leu-Thr-Asn-Lys-Asn-Val-Ile-
11 Phe-Val-Ala-Gly-Leu-Gly-Gly-Ile-Gly-Leu-
21 Asp-Thr-Ser-Lys-Glu-Leu-Leu-Lys-Arg-Asp-
31 Leu-Lys-Asn-Leu-Val-Ile-Leu-Asp-Arg-Ile-
41 Glu-Asn-Pro-Ala-Ala-Ile-Ala-Glu-Leu-Lys-
51 Ala-Ile-Asn-Pro-Lys-Val-Thr-Val-Thr-Phe-
61 Tyr-Pro-Tyr-Asp-Val-Thr-Val-Pro-Ile-Ala-
71 Glu-Thr-Thr-Lys-Leu-Leu-Lys-Thr-Ile-Phe-
81 Ala-Gln-Leu-Lys-Thr-Val-Asp-Val-Leu-Ile-
91 Asn-Gly-Ala-Gly-Ile-Leu-Asp-Asp-His-Gln-
101 Ile-Glu-Arg-Thr-Ile-Ala-Val-Asn-Tyr-Thr-
111 Gly-Leu-Val-Asn-Thr-Thr-Thr-Ala-Ile-Leu-
121 Asp-Phe-Trp-Asp-Lys-Arg-Lys-Gly-Gly-Pro-
131 Gly-Gly-Ile-Ile-Cys-Asn-Ile-Gly-Ser-Val-
141 Thr-Gly-Phe-Asn-Ala-Ile-Tyr-Gln-Val-Pro-
151 Val-Tyr-Ser-Gly-Thr-Lys-Ala-Ala-Val-Val-
161 Asn-Phe-Thr-Ser-Ser-Leu-Ala-Lys-Leu-Ala-
171 Pro-Ile-Thr-Gly-Val-Thr-Ala-Tyr-Thr-Val-
181 Asn-Pro-Gly-Ile-Thr-Arg-Thr-Thr-Leu-Val-
191 His-Lys-Phe-Asn-Ser-Trp-Leu-Asp-Val-Glu-
201 Pro-Gln-Val-Ala-Glu-Lys-Leu-Leu-Ala-His-
211 Pro-Thr-Gln-Pro-Ser-Leu-Ala-Cys-Ala-Glu-
221 Asn-Phe-Val-Lys-Ala-Ile-Glu-Leu-Asn-Gln-
231 Asn-Gly-Ala-Ile-Trp-Lys-Leu-Asp-Leu-Gly-

241 Thr-Leu-Glu-Ala-Ile-Gln-Trp-Thr-Lys-His-

251 Trp-Asp-Ser-Gly-Ile

Appendix II

Secondary Structure Prediction for DmADH by the Computerized Method of Chou and Fasman



* This figure is adapted from Benyajati *et al.* (1981).

Appendix III

THE SEQUENCES OF SYNTHETIC OLIGONUCLEOTIDES

Synthetic oligonucleotides used as primers for mutagenesis and DNA sequencing.

Code	Sequence (5'-3')	Start position*(nt)	Remarks
IntronI	ACGAGTTCGCGCTAGACTTC TTGGACCACTAGGAGCTGGC	79	Intron deletion
IntronII	TGAAGTGGTCGAGGGACCGC TTTGACCGGGGGTAATGGCC	549	Intron deletion
M13AC	ACATCGATCTGCAGG		To mutate *Acc*I to *Cla*I in M13 polylinker
ADHECO	ATGGTGAATTCTTTTGC	-17	To create *Eco*RI site before the starting codon
ADHA14	CTCCCAGAGCGGCAACG	36	To mutate G14 to A14
ADHV14	CTCCCAGAACGGCAACG	36	To mutate G14 to V14
ADHA135	CGGATCCAATGTTGGCGATG ATACCACC	458	To mutate C135 to A135
ADHA218A	ACGAAGTTCTCGGCAGCCGC CAACGATGGCT	775	To mutate C218 to A218 by PCR
ADHA218B	AGCCATCGTTGGCGGCTGCC GAGAACTTCGT	775	To mutate C218 to A218 by PCR
ADHBAMA	ATCTGCAACATTGGATCCGT	467	To keep *Bam*HI site in DmADH gene
M13ECOB	AAAACGACGGCCAGTGAATT		To keep *Eco*RI site in M13 polylinker
ADH2	CTTCAGATCGCGCTT	84	Sequencing primer
ADH3	CAATGGGCACGGTCACAT	258	Sequencing primer
ADH4	AATGGTGCGCTCGATCTG	365	Sequencing primer
ADH5	CATTGAATCCAGTGACGG	483	Sequencing primer

ADH6	GTTCACGGTGTAAGCGGT	663	Sequencing primer
ADH7	GGCGCAGGCCAACGATGG	777	Sequencing primer
ADH8	CCAGTGCTTGGTCCACAG	873	Sequencing primer
ADH9	CTATGAACTAATGTTATG	931	Sequencing primer
ADH10	GGATTTCTCAATTTTCATA	1061	Sequencing primer

* Position No.1 is base A in ATG starting codon for DmADH.

Appendix IV

COMMON METHODS

The common solutions (including their definitions and compositions) and reagents used in the following procedure will be listed in Appendix V.

A. Site-directed Mutagenesis - Kunkel's Method

The major procedure of this method has been described by Kunkel *et al.* (1987).

I. Preparation of Uracil-Containing Template

1. Inoculate two tubes containing 2.5 ml of YT medium with an individual colony of BW313 and grow for 6-8 hours.
2. Prepare 100 ml YT in a 1000 ml flask with 0.25 µg/ml Uridine.
3. Add mid-log cells (5 ml) to the YT culture.
4. Centrifuge the M13 phage stock for 1 min, then add 100 µl to the YT culture.
5. Grow at 37°C overnight.
6. Centrifuge 5 min at 12,000 x g (9,000 rpm by JM14 Rotor or equivalent).
7. Pour the supernatant to a clean tube.
8. Add 25 ml 20% PEG/2.5 M NaCl, vortex well, and leave at room temperature for 1 hours.

9. Centrifuge at the same speed for 5 min and remove PEG completely.
10. Resuspend the phage pellet in 2 ml TE (pH 8.0).
11. Add equal volume of buffer saturated phenol and vortex well. Keep at room temperature for 5 min and spin 2 min to separate two phases, then remove the upper aqueous phases.
12. Add equal volume of chloroform and vortex well. Keep at room temperature for 5 min and spin 2 min to separate two phases.
13. Remove the upper aqueous phase. Add 1/10 volume of NaOAc (3M) and 2.5 volume of ethanol. Precipitate at -70°C for an hour.
14. Centrifuge 12,000 x g (10,000 rpm, JM20 rotor or equivalent) at 4°C for 30 min. Carefully pour off the ethanol.
15. Wash with 70% ethanol and dry DNA pellet under vacuum.
16. Dissolve the pellet in 0.5 ml TE (pH 8.0).

II. ***In Vitro* Mutagenesis**

1. Phosphorylation of mutagenic primer

Kinase reaction buffer 10X	2 µl
ATP (10 mM)	2 µl
Mutagenic primer (1.5 pmole)	x µl
Distilled H_2O	y µl
T4 DNA kinase (3 u/µl)	1 µl
Total Volume:	20 µl

Incubate the reaction mixture at 37°C for 1 hour and stop by adding 0.6 µl EDTA (0.5 M).

2. Hybridization of phosphorylated primer to the uracil-containing template

To the kinase reaction mixture, add 0.3 pmole uracil-containing template and 1.2X SSC. Heat it in 65°C water bath for 30 min, then cool down to room temperature slowly. Keep the reaction mixture on ice.

3. In *Vitro* DNA synthesis

To the annealed template, add:

Hepes (0.1 mM, pH 7.8)	20	µl	
DTT (1 M)	0.5	µl	
$MgCl_2$ (1 M)	1	µl	
dNTP (100 mM)	0.5	µl	(for each)
ATP (10 mM)	1	µl	
DNA polymerase (Klenow) (5 u/µl)	0.5	µl	
T4 polynucleotide Ligase (1.4 u/µl)	2	µl	
Distilled H_2O	x	µl	
Total Volume:	100	µl	

Keep the above mixture in ice bath for 5 min. Keep in room temperature for 5 min, then incubate at 37°C for 2 hours. Stop the reaction by adding 3 µl EDTA (0.5 M).

4. Product analysis

Load 20 µl of the reaction solution on 0.8% TAE agarose gel. Run the electrophoresis at 20 volts for overnight. The known single strand DNA and

for overnight. The known single strand DNA and double strand DNA can be used as markers.

III. **Preparation of Competent Cells and Transformation**

The transformation technique has been discussed by Hanahan (1983).

1. Dilute the overnight CSH50 cell culture (YT) by ratio of 1:100 into a 50 ml YT medium and grow at 37°C for 2.5 hours.
2. Cool the cells on ice for 15 min.
3. Spin 25 ml at scale 7 for 15 min in cold room.
4. Resuspend in 8 ml ice cold TFB and place on ice for 30 min.
5. Repeat step 3.
6. Resuspend in 2 ml cold TFB.
7. Add 70 µl of DMSO and keep on ice for 5 min.
8. Add 70 µl of DTT (2.25 M) and keep on ice for 10 min.
9. Repeat step 7.
10. Transfer 200 µl competent cells to each sample tube.
11. Add 1 µl the diluted (1:5 by TE) reaction mixture from *in vitro* mutagenesis step II-3 to competent cells.
12. Incubate on ice for 30 min.
13. Heat shock cells at 42°C for 90 sec and chill on ice.

14. Mix the sample with 3 ml soft agar (at 47°C) containing 50 µl 2% x-gal and 10 µl 100 mM IPTG , pour the mixture immediately onto a YT plate, and incubate at 37°C overnight.

IV. Plaque Hybridization and Screening of Mutations

The major steps of this protocol is described by Seong & RajBhandary (1987).

1. Choose a plate that has 100-200 plaques and chill for 30 min at 4°C.
2. Lay a dry nitrocellulose filter onto the agar and let the phage adsorb for 5 min.
3. Mark the filter by stabbing an inked needle through filter.
4. Carefully peel the filter and air dry the filter for 5 min on a sheet of Whatman 3 MM.
5. Wet a sheet 3MM paper with 0.5 N NaOH/1.5 M NaCl. Roll off the excess liquid with a pipet.
6. Place the filter onto the paper with plaques facing up and stand for 5 min. Remove and air dry for 2 min.
7. Repeat step 6.
8. Place the filter onto a sheet of 3MM paper soaked with 1 M Tris-Cl (pH 8.0) and let stand for 5 min. Remove and dry 2 min.
9. Repeat step 8.

11. Wash the filter in 100 ml 6X SSC for 2 min and air dry.
12. Bake in vacuum oven for 1 hour at 80°C.
13. Place the filter (plaques facing up) into the petri-dish filled with 5 ml pre-hybridization solution.
14. Swirl to completely wet the filter, cover and incubate at 37°C for 1 hour.
15. Pour off pre-hybridization solution and add 5 ml probe (^{32}P labeled mutagenic primer) solution in the same petri-dish and incubate at room temperature overnight.
16. Remove the probe solution and save it in -20°C.
17. Wash the filter in 100 ml 6X SSC for 15 min, repeating 3 times at room temperature.
18. Wrap the filter in saran wrap and develop autoradiography for 3 hours with an intensifier at -70°C.
19. Briefly wash in 3 M $(CH_3)_4NCl$ at room temperature about 3 min. Wash the filter in an appropriate temperature [4-5°C below Td in $(CH_3)_4NCl$].
20. Remove the filter and check the signal with Geiger counter, if the signal is still high, then raise the temperature by 2°C and wash it again. Repeat washing until the signal become weak.
21. Air dry the filter and autoradiography overnight.

22. Comparing the film developed before washing with the one after washing, the mutation should be identified.

In addition to this method, a mutation can also be identified by DNA sequencing (see Appendix Four, Section F).

B. **Site-directed Mutagenesis - Polymerase Chain Reaction (PCR)**

These procedures are modified methods developed by Higuchi *et al.* (1988) and Perkin-Elmer Cetus in the catalog of "GeneAmp DNA Amplification Reagent Kit".

I. **Producing two DNA fragments with the same mutation site which is at 3′ end of one fragment (fragment A) and 5′ end of another (fragment B).**

1. Purify two pairs of synthetic primers (A-3′& A-5′, B-3′ & B-5′). Their purification will be described in Appendix IV section E.
2. The reaction mixture:

Reaction buffer 10X	25	µl
dNTPs (1.25 mM)	40	µl
Primer 1 (A-3′ or B-3′) (50 pmole)	x	µl
Primer 2 (A-5′ or B-5′) (50 pmole)	y	µl
Template DNA (0.3 pmole)	z	µl
Double-distilled H_2O	w	µl
Taq DNA polymerase (5 u/µl)	1.3	µl
Total Volume:	250	µl

3. PCR reactions are performed in a Perkin-Elmer Cetus DNA Thermal Cycler. Each cycle consists of denaturation at 94°C for 2 min, annealing at 37°C for 1 min, and extension at 72°C for 2.5 min. Twenty cycles are performed. Extension time of the last cycle is 10 min to ensure that all single stranded DNA have been copied or re-annealed to form double strands.
4. The products are extracted once with 100 µl chloroform.
5. The two DNA fragments produced are purified by agarose gel electrophoresis followed by elution as described by Maniatis *et al.* (1982).
6. Extract the eluate with water saturated 1-butanol once and ethanol precipitate at -70°C overnight.
7. Wash with 70% of ethanol and dry DNA pellets.
8. Dissolve the pellets in 50 µl TE (pH 8.0).

II. **Connection of the two DNA fragments at mutation site by the second round of PCR**

1. Mix the two DNA fragments with the primer A-5′ and B-3′. The reaction conditions are exactly the same as the step I-3.
2. Extract the reaction mixture with chloroform once.
3. Extract the reaction mixture with phenol once.
4. Precipitate with ethanol at -70°C overnight.
5. Wash with 70% of ethanol and dry the pellet.

6. Dissolve in 10 µl TE (pH 8.0) and check the size and purity of produced DNA by agarose gel electrophoresis.
7. The PCR produced DNA fragment which contains a desired mutation can be linked to a DNA vector by appropriate restriction sites.

C. Preparation of Plasmid and Phage DNA Vector

I. Mini-preparation Procedure

This protocol was similar to the alkaline lysis method described by Maniatis *et al.* (1982).

1. Pour 1.5 ml of over night cell culture [*E. coli* with plasmid or infected with bacteria phage (for M13, see sequencing template preparation)] into an eppendorf tube. Centrifuge for 5 min at room temperature.
2. Drain the supernatant, leaving the pellet as dry as possible.
3. Resuspent the pellet in 100 µl of ice-cold solution I and keep at room temperature for 5 min.
4. Add 200 µl of fresh made ice-cold solution II. Mix the contents by inverting the tube rapidly. Do not vortex. Keep it on ice for 5 min.
5. Add 150 µl of ice-cold solution III. Mix it by inverting tube rapidly for five times. Keep it on ice for 10 min.

6. Centrifuge at 4°C for 10 min and transfer the supernatant to a fresh tube.
7. Add 0.5 ml of phenol/chloroform (1:1), vortex, and stand for 2 min. Centrifuge at room temperature for 2 min and transfer the supernatant to a fresh tube.
8. Add 1 ml ethanol at room temperature, keep it at room temperature for 5 min, and centrifuge at room temperature for 5 min.
9. Remove the supernatant. Stand the tube in an inverted position on a napkin to allow all of the fluid to draw away.
10. Wash the pellet with 1 ml of 70% ethanol and re-centrifuge at room temperature for 5 min.
11. Remove ethanol and dry the pellet briefly by vacuum.
12. Dissolve the DNA in 60 µl TE (pH 8.0). Take 10 µl for digestion assay.

II. **Large-Scale Preparation without using CsCl centrifugation**

This protocol is the modified protocol from Barttlet (personal communication).

1. Inoculate 2.5 ml LB broth containing an appropriate antibiotic or 2.5 ml YT with a single colony, and grow 8-12 hours.
2. Transfer 2.5 ml liquid cell culture (LB or YT) to 250 ml LB with a same antibiotic or 250 ml YT plus 500 µl M13 stock and incubate it at certain temperature (37°C or otherwise specified overnight.

3. Chill the culture for 15 min.
4. Pellet cells 6,000 x g (5000 rpm, JM14 rotor or equivalent) at 4°C for 15 min.
5. Resuspend the cell pellet in 5 ml solution I, then transfer to an 40 ml tube.
6. Add 1 ml lysozyme solution (12 mg/ml in solution I) to the final concentration of 2 mg/ml. Incubate on ice for 20 min.
7. Add 12 ml ice cold solution II and incubate on ice for 10 min.
8. Add 8 ml solution III and stand on ice for 20 min.
9. Centrifuge 39,100 x g (18,000 rpm, JM20 rotor or equivalent) for 30 min at 4°C and transfer the supernatant to two 50 ml Polypropylene Centrifuge Tubes.
10. To each Polypropylene Centrifuge Tube, add 5 µl (10 mg/ml) DNase-free RNase A. Incubate at 37°C for 40 min.
11. Extract each tube twice with equal volume of phenol/chloroform (1:1, v/v). Spin in large bench centrifuge for 15 min.
12. Transfer the aqueous phase to Corex tubes. Add two volumes of ethanol. Precipitate DNA on ice for 30 min.
13. Spin 12,000 x g (10,000 rpm, JM20 rotor or equivalent) at 4°C for 30 min.

14. Dissolve the pellets in total 1 ml double distilled H_2O, then wash all the tubes by 0.6 ml double distilled H_2O. To total 3.2 ml add 0.64 ml 5 M NaCl and 4 ml 13% PEG and incubation on ice for 1 hour.
15. Pellet DNA by centrifuge 12,000 x g (10,000 rpm, JM20 rotor or equivalent) at 4°C for 30 min.
16. Wash pellet with 70% ethanol.
17. Completely dry the pellet under vacuum and dissolve DNA in TE (pH 8.0).

D. **Subcloning of DNA Fragments**

I. Isolate the DNA fragment from low melting gel

1. Prepare 1% low melting gel (SeaPlaque) in 50 mM Tris-acetate (TAE) buffer. It should be cooled to 37°C before pouring.
2. After sample DNA and vector are digested with certain enzymes, inactivate the enzymes by heating or EDTA and keep in ice if they are heat treated.
3. Prior to loading the sample the gel should be pre-run for at least 10 min. During this pre-run the voltage should be slowly increased to the required setting (5-7 V/cm).
4. After electrophoresis of the DNA, specific fragments can be localized by staining the gel for 10 min with 0.2 µg/ml ethidium bromide (2,7-Diamino-10-ethyl-9-phenylphenanthridium bromide) and visualized with long wave length UV light.

II. Ligation reaction

1. Gel slices containing specific fragments can be cut out and melted at 70°C for 10 min and kept in 37°C.

2. Protocol for ligation reaction:

Vector DNA	x µl	(mole of Vector : mole of Insert = 1:2)
insert DNA	y µl	(x + y = 10 µl)
double distilled H_2O	5 µl	
10x ligation buffer	4 µl	
T4 DNA ligase (2 u/µl)	1 µl	
Total Volume:	20 µl	

The reaction mixture is incubated at 15°C for 12-20 hours.

III. **Transformation (BRL protocol #8264SA for DH5αF' competent cell)**

1. Remove competent cells and lawn cells from freezer; thaw on ice.
2. Transfer 50 µl to a polypropylene tubes and the remaining should be refreezed in the dry ice/ethanol bath for 5 min, then returned to -70°C freezer.
3. Dilute the ligation mixture 5 times with TE (pH 8.0) and transfer 1 µl to competent cells.
4. Mix them gently and incubate on ice for 30 min.
5. Heat-shock cells 45 sec in a 42°C water bath.
6. place on ice for 2 min.
7. Add the transformed competent cells to 3 ml top agar (YT or LB) containing 50 µl lawn cells, 50 µl 2% x-gal and 10 µl 100 mM isopropyl- -D-hiogalacto-

pyranoside (IPTG) (x-gal and IPTG are required only for the vector containing β-glacosidaes gene) and plate on agar plates.

8. Incubate the plate at 37°C overnight.
9. Identify the correct colonies by mini-preparation of DNA followed by restriction enzyme digestion and gel electrophoresis.

E. Purification of Synthetic Oligonucleoside

This is a modified protocol described in the catalog of "Oligonucleoside Purification Cartridges" from Applied Biosystems.

1. Flush the cartridge with 5 ml HPLC grade acetonitrile, followed by 5 ml 2.0 M triethylamine acetate. Keep the flow rate at 1 drop/second.
2. Load the crude synthetic oligonucleoside into the syringe which is connected to the cartridge and then gently push through the cartridge. Save the eluted fraction and reload this fraction second time.
3. Flush cartridge with 5 ml diluted ammonium hydroxide (1:10 dilution of 30% pure ammonium hydroxide in deionized water) for 3 times.
4. Wash cartridge with 5 ml deionized water for twice.
5. Detritylated the support-bound oligonucleoside with 5 ml of 2% trifluoroacetic acid solution twice.
6. Flush cartridge with 5 ml deionized water twice.

7. Elute the purified, detritylated oligonucleoside by flushing the cartridge with 3 ml of the 20% acetonitrile solution.
8. Evaporate to dryness an aliquot of the eluate and dissolved in 2X TE (pH 8.0).
9. The purity of the oligonucleoside can be checked by kinase reaction with ^{32}P and autoradiography.

F. DNA sequencing

I. Preparation of Template DNA

This protocol was described by Sanger *et al.* (1977). It has been used to prepare template DNA for sequencing. The DNA template prepared from this procedure is sufficiently pure for sequencing uses.

1. Inoculate one tube containing 2 ml of YT medium with a single colony of DH5 F' and grow at 37°C for overnight
2. Transfer 30 µl overnight cell culture to 3 ml YT and add 7.5 µl supernatant of stock phage culture to the same YT containing tube.
3. Grow cells at 37°C for 5.5 hours with shaking.
4. Transfer 1.5 ml of the culture to an eppendorf tube and centrifuge for 5 min.
5. Pour the supernatant to a clean eppendorf tube taking care not to carry over any cells and add 200 µl of 20% PEG/2.5 M NaCl.

6. Vortex the supernatant well and leave them at room temperature for 30 min.
7. Centrifuge the tubes for 5 min and remove all residual supernatant completely.
8. Resuspend the phage pellets and combine two tubes in 100 µl of TE (pH 8.0).
9. Add an equal volume of buffer saturated phenol, vortex well, and leave for 5 min. Vortex and centrifuge for 2 min.
10. Transfer upper aqueous layer to a new tube and add 1/10 volume of NaOAc (3 M), 2.5 volume of ethanol and precipitate at -70°C for 30 min.
11. Centrifuge for 30 min at 4°C and carefully pour off the ethanol.
12. Wash the pellet with 70% ethanol and vacuum dry.
13. Redissolve the pellet in 25 µl of TE (pH 8.0) and store at -20°C.
14. Take 3 µl to run a 0.8% agarose gel to check the purity and concentration using a known concentration phage DNA as the standard.

II. **Preparation of 6% Polyacrylamide Gel**

This procedure is modified from M13 cloning and sequencing manual (BRL).

1. Carefully clean one pair of glass gel plates with detergent.

2. Siliconize (Sigma-coat) the inside of the smaller plate by wiping with glass wool socked with 5% solution of dichlorodimethylsilane in chloroform and allow to air dry. Wash away the remaining siliconizing solution with water and dry the plate again. The siliconized plate is good for at least 5 uses.
3. Right before use, wipe clean with 95% ethanol.
4. Assemble the glass plate using side spacers and bulldog clamps and keep it horizontally.
5. For 50 cm x 20 cm gel, measure 80 ml of 6% acrylamide stock solution and degas for 15 min.
6. Initiate polymerization by adding 45 µl TEMED and 240 µl 10% ammonium persulfate.
7. Mix the acrylamide solution well and pour to the plate as quick as possible. Put the comb in place. The gel should polymerize after 5 min and within 20 min.
8. Cover both top and bottom of the gel with saran wrap and store it for overnight.

III. **Dideoxy Sequencing Reaction**

Routinely a Sequenase Kit purchased from USB INC is used. The following protocol is described in USB procedure.

1. Annealing Mixture

For each sample:

Template DNA (1-2 µg)	x µl
Primer DNA (2.5-3 ng)	y µl
distilled H_2O	z µl
Buffer 5x	2 µl
Total Volume:	10 µl

Anneal by heating the mixture at 70°C water and cool slowly to < 35°C.

2. While cooling, fill the pre-labeled tube with 2.5 µl of each Termination Mixture (ddNTP) and pre-warm at 37°C water bath.

3. Dilute the Labeling Mix 1 : 5 by distilled H_2O.

4. Pre-cool TE (pH 7.5) for dilution of sequenase.

5. Labeling Reaction

To Annealed DNA Mixture (10 µl), add

DTT (0.1 M)	1	µl
Diluted labeling mix	2	µl
[^{35}S] dATP	0.5	µl
Diluted Sequenase	2	µl
Total Volume:	15.5	µl

Incubate at room temperature for 5 min.

6. Termination Reaction

Transfer 3.5 µl of labeling reaction to each termination mixture and incubate at 37°C for 5 min.

7. Stop by adding 4 µl stop solution. The sequencing mixture can be store at -20°C for a few weeks.

IV. Gel Electrophoresis and Post-treatment

1. Pre-run the gel at 45 watts for 30 min.

2. Heat the samples in a 90°C water bath for 5 min, then quickly load appropriate amount to the gel. Run at the same watts until the fast dye reaches the bottom of gel.
3. Carefully separate two glass plates using spatula, leave gel lying on the larger plate.
4. Fix the gel in 10% Methanol/10% Acetic Acid for 30 min.
5. Cover the gel with two sheets of 3MM paper and invert the glass plate to transfer the gel to the 3MM paper.
6. Cover the gel with saran wrap and dry the gel at 80°C for 0.5-1.0 hours on a gel dryer.
7. Place a sheet of X-ray film on top of the gel and expose at room temperature overnight.

G. Purification of *Drosophila* ADH from *E. coli* Host

I. Bacteria Cell Lysis

1. One liter of Transformed cells at late log phase are pelleted at 4°C, 7,000 x g (7,000 rpm, JM14 rotor or equivalent), 30 min and resuspended at 10% by weight in sonication buffer.
2. Sonication is performed on ice, using a Horn tip at level 6, pulse, and 50%. The cell solution is sonicated for 2 min, cooled for 2 min, and then the same action is repeated twice.

3. Spin the cells at 27,000 x g (15,000 rpm, JM20 rotor or equivalent) at 4°C for 30 min.
4. Resuspend the pellet in 10-15 ml sonication buffer and repeat the sonication again.
5. Repeat step 3 and measure the final volume of the supernatant.

II. **Ammonium sulfate precipitation**

1. To the crude extract, add solid $(NH_4)_2SO_4$ very slowly to 40% saturation and allow 15 min on ice.
2. Centrifuge 27,000 x g (15,000 rpm, JM20 rotor or equivalent) and measure the volume of supernatant.
3. To the supernatant, add solid $(NH_4)_2SO_4$ very slowly to 60% saturation and keep it at 4°C for at least 2 hours.

III. **Sephadex G-100 Column**

1. A Sephadex G-100 column (size: 2.5 x 120 cm) has been packed one day before running, using column buffer saturated and degassed Sephadex G-100 (40 g).
2. The column should be pre-equilibrated at running flow rate (30 ml/hr) overnight in cold room.
3. Check the conductivities of the equilibration buffer and eluate to determine if the column is equilibrated.
4. Calibrate the column by 2 ml blue dextran (2 mg/ml).

5. The precipitated protein is centrifuged at 27,000 x g (15,000 rpm, JM20 rotor or equivalent) for 30 min and dissolved in 5 ml column running buffer.
6. Load the protein onto the column and allow it runs into Sephadex. Wash the column with column running buffer.
7. Collect the eluate and measure protein concentration by UV absorbance at 280 nm.
8. Each collected eluate (about 5 ml) is also assayed for ADH activity (see Appendix IV-H).
9. Eluted fractions containing the majority of ADH activity are pooled and applied to a Cibacron Blue 3GA-Agarose column.

IV. Cibacron Blue 3GA-Agarose Column

1. Cibacron Blue 3GA-Agarose (20 g) is suspended in column running buffer and gently stirred at 4°C to break any clumps for at least 1 hour.
2. Transfer the resin to the column (2.5 x 20 cm) without trapping air bubbles.
3. The column is equilibrated at 4°C with column running buffer by the flow rate 24 ml/hour overnight.
4. Check the conductivities of the equilibration buffer and the elate to determine if the resin is equilibrated.

5. Load the protein onto the column and wash with column running buffer until the absorbance of the eluate at 280 nm is almost zero.
6. Use a BRL gradient former to make a linear NaCl gradient (0 - 2 M). The inner cylinder of the gradient former is filled with 200 ml column buffer and the outer is filled with the same buffer containing 2 M NaCl.
7. Elute proteins by the linear NaCl gradient, collect eluate, and assay for ADH activity.
8. The fraction which contain the majority of ADH activity can be dialyzed and concentrated in an Amicon ultrafiltration cell.
9. The purity of the collected ADH protein is determined by using a 10% SDS-PAGE.

H. **ADH Activity Assay**

The assay condition is designed by Lee (1982).

Assay solution (1 ml):

Tris.Cl (pH 9.8) (1 mM)	100	µl
NAD (100 mM)	10	µl
Alcohol (d=0.78 g/ml)	64	µl
Double distilled H_2O	x	µl
ADH protein	y	µl
Total Volume:	1000	µl

The reaction is started by adding ADH and the absorbance at 340 nm is recorded by spectrophotometer (Gilford-Response II). One unit of activity is defined as a change in absorbance at 340 nm of 0.001 per min (Sofer & Ursprung, 1968).

I. Protein Assay

The protein assays in this project are performed by using Bio-Rad protein assay solution. The principle and reagent of the assay solution are described in Bio-Rad catalog.

1. Dilute Bio-Rad protein assay solution by distilled H_2O (1 : 5, v/v).
2. Make assay standards by BSA of known concentration (2, 4, 6, 8, and 10 µg/100 µl). Add each of the standard BSA of 100 µl to 5 ml diluted assay solution and take 1 ml for the assay.
3. Record the absorbance at 595 nm of each standard to create a standard curve on spectrophotometer (Gilford-Response II).
4. Assay unknown proteins at the same conditions as the BSA standards.

J. Western Blot Protein Analysis

The procedure of western blot on ADH has been described by Batzer *et al.* (1988).

1. Make up 4 liters of transfer buffer and keep it at 4°C.
2. Saturate the filter paper, sponge pads, and blotting membranae in transfer buffer for 30 min.
3. Saturate SDS-PAGE in transfer buffer for 30 min.
4. In blot holder, layer the following in the order of sponge pad, filter paper, SDS-PAGE, blotting

membranae, filter, and sponge pad. Insert them into apparatus so that blotting membranae side is toward the anode.

5. Fill the apparatus with transfer buffer and run at 30 volts for 7 hours.
6. Sock the membranae in blocking solution at 4°C overnight.
7. Incubate blotting membrane with anti-ADH antibody that is diluted (1:10) with blocking solution (about 5 ml) at 4°C overnight.
8. Wash membrane 6 times with Dubiccos phosphate buffer. Each wash for at least 15 min at room temperature.
9. Incubate blotting membrane with alkaline phosphatase conjugated anti-rabbit IgG that is diluted (1:1000) in blocking solution (250 ml). Leave it overnight at 4°C.
10. Wash the membrane 6 times with Dubicoos phosphate buffer. Each for at least 15 min at room temperature.
11. Incubate the membrane with substrate solution. Starts visualization in about 10 min. When completely developed pour away substrate solution and put the membrane in distilled water.
12. Dry the membrane at room temperature.

Appendix V

Common Solutions/Reagents

Bacteria Mediums

LB (Luria-Bertani)	per liter	Bacto-tryptone	10 g
		Bacto-yeast	5 g
		NaCl	5 g
	Adjust pH to 7.5.		
	for plate	Bacto-agar	15 g
	for top agar	bacto-agar	7 g
YT Medium	per liter	Bacto-tryptone	8 g
		Bacto-yeast	5 g
		NaCl	5 g
	Adjust pH to 7.5.		
	for plate	Bact-agar	15 g
	for top agar	Bact-agar	7 g

DNA buffers

TE (Tris-EDTA)	10 mM Tris-Cl (pH 8.0)		
	1 mM EDTA		
TBE (Tris-Borate-EDTA)	Per liter 10x stock solution		
	Tris base	121.1	g
	Boric acid	55	g
	EDTA	7.4	g
	Adjust pH to 8.0.		
TAE (Tris-Acetate-EDTA)	Per liter 50x stock solution		
	Tris base	242	g
	Glacial acetic acid	57.1	ml
	0.5 M EDTA (pH 8.0)	100	ml
	Adjust pH to 8.0.		

Transformation reagents

100 mM IPTG	23.8 mg IPTG/ml in H_2O (MW. 238.3), store at -20°C.

X-gal 2% solution	25 mg in 1.25 ml DMSO, store at 4°C in a dark bottle.
1 M K-MES (pH 6.3)	9.76 g in 50 ml H_2O (MW. 195.2) using 10 M KOH to adjust pH to 6.3, filter, store at -20°C.
TFB (Transformation buffer)	10 mM K-MES (pH 6.3), 100 mM RbCl, 45 mM $MnCl_2.4H_2O$, 10 mM $CaCl_2.2H_2O$, 3 mM $HACoCl_3$, filter, store at 4°C.
2.25 M DTT	347 mg in 1 ml 40 mM KAC (pH 6.0) (MW. 154.3), filter, store at -20°C.

Plaque hybridization solutions

20 X SSC	175.3 g of NaCl and 88.2 g of Tri-sodium citrate in 800 ml of H_2O. Adjust pH to 7.0 with a few drops of HCl. Adjust volume to 1 liter.
50 X Denhart's solution	For 500 ml stock solution Ficoll 5 g PVP 5 g BSA (Pentax Fraction V) 5 g --- Add H_2O to 500 ml Filter through a Nalgene filter. Dispense into 25 ml aliquots and store at -20°C.
Prehybridization solution	6 X SSC, 10 X Denhart's, and 0.1 % SDS.
Hybridization solution	6 X SSC, 10 X Denhart's, and 5 x 10^7 cpm probe.

Plasmid preparation solutions

Solution I	50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA.
Solution II	0.2 N NaOH, 1 % SDS (use and made freshly)
Solution III	To 60 ml of 5 M potassium acetate add 11.5 (5 M KOAc) ml of glacial acetic acid and 28.5 ml H_2O. The resulting solution is 3 M with respect to acetate (pH 4.8).

Polyacrylamide gel electrophoresis (PAGE)

The following PAGEs are prepared from 40% acrylamide (ACRYL-40) and 2 % bis-acrylamide (BIS-2) stock solutions (American Research Products Company).

6% Sequencing gel (Stock Solution)	per	250 ml	
		ACRYL-40	35.6 ml
		BIS-2	37.5 ml
		urea	115 g
		10 X TBE	12.5 ml
	Add	H_2O to	250 ml
20 % Oligomer purification gel	per	120 ml	
		ACRY-40	57 ml
		BIS-2	60 ml
		urea	20 g
		10 X TBE	12 ml
	Add	H_2O to	250 ml
	degas 15 min		
	Add	10 % Ammonium persulfate	300 µl
		TEMED	60 µl
10 % SDS protein gel	per	50 ml	
		ACRY-40	12.5 ml
		BIS-2	5 ml
		urea	1.5 g
		10 % SDS	0.5 ml
		1 M phosphate	5 ml
	Add	H_2O to	50 ml
		10 % Ammonium persulfate	260 µl
		TEMED	65 µl
SDS-PAGE running buffer	0.1 M phosphate (pH 7.4), 0.1 % SDS, and 1.76 ml of thiolatic acid. Adjust pH to 7.4 by NaOH and add H_2O to 2 liters.		
SDS-PAGE fixing solution	50 % methanol and 10 % acetic acid		

SDS-PAGE staining solution	per 500 ml coomaisse brillant blue R-250 1.25 g acetic acid 21 ml methanol 227 ml H_2O 227 ml --- Stirring for 1 hour and filtrate through whatman filter.
SDS-PAGE destaining solution	25 % methanol and 7.5 % acetic acid
5 X SDS-PAGE sample buffer	300 mM phosphate buffer (pH 7.4), 50 % glycerol, 250 mM DTT, 0.125% BPB, and 10 % SDS.
Cellophane treatment solution	5 % $NaCO_2$ and 50 mM EDTA. Boil for 5 min and rinse in distilled H_2O.

Protein purification solutions

Sonication buffer	50 mM phosphate, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF (0.2 M stock solution in ethanol)(pH 7.4).
Column buffer	50 mM tris-Cl and 1 mM EDTA (pH 8.3).

Western blot solutions

Transfer buffer	2 mM sodium acetate, 5 mM MOPS (pH 7.5), and 20 % (v/v) ethanol.
10 X Dulbiccos	For 1 liter KCl 2 g KH_2PO_4 2 g NaCl 80 g Na_2HPO_4 11.3 g (0.08 M) Sodium Azide 1 g
Blocking solution (1 liter)	1 X Dulbiccos buffer, 50 g Carnation non-fat dry milk, and 0.5 g sodium azide.

Substrate solution	1. 50 µl 5-bromo-4-chloro-3-indolyl (BCIP) stock solution. (The stock solution is 100 mg/ml BCIP in imethyl-sulfoxide and stored at -20°C). 2. 10 ml nitro blue tetrazolium (NBT) stock solution (The stock solution is 1 mg/ml NBT in H_2O stored at 4°C). 3. 90 ml tris buffer (100 mM tris, pH 8.8; 1 mM $MgCl_2$).

Appendix VI

*Dm*Adh Sequence and Endonuclease Cutting Sites

This gene map was generated from the Sequence Analysis Software Package of the Genetic Computer Group (GCG) (University of Wisconsin Biotechnology Center).

KEYWORDS alcohol dehydrogenase; dehydrogenase.
SOURCE drosophila melanogaster (adh-s and adh-f) cdna ([1]) and dna . . .

With 149 enzymes: *

• July 7, 1989 17:13 ..

```
                                A
                                s
   H                            p                          M
   i                           S7                          a
   n                           s0                          e
   f                           p0                          I
   I                           II                          I
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                    B                                B   T
                    s                                s   t
                    p                                p   h
             A      1H                           C   B1  1
             p      2g                           v   a2M 1
             a      8i                           i   n8s 1
             L      6A                           J   I6e I
             I      II                           I   III I
                     /                                /
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     T
     t
     h S            H
     1 a            i
     1 u D          nFH                              B  B  B  F
     1 3 p          Psh                              b  b  b  o
     I A n          lpa                              v  v  v  k
     I I I          III                              I  I  I  I
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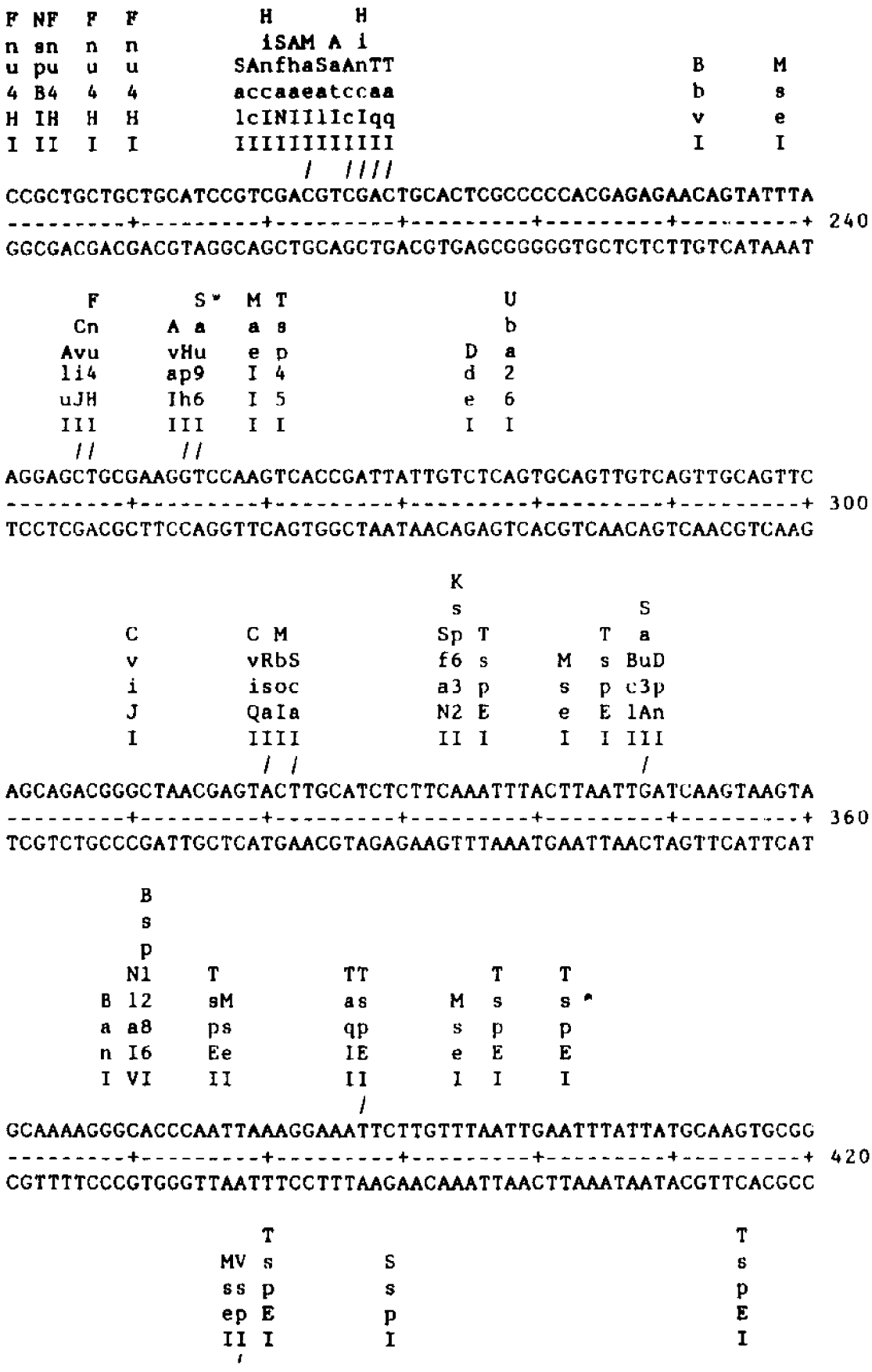
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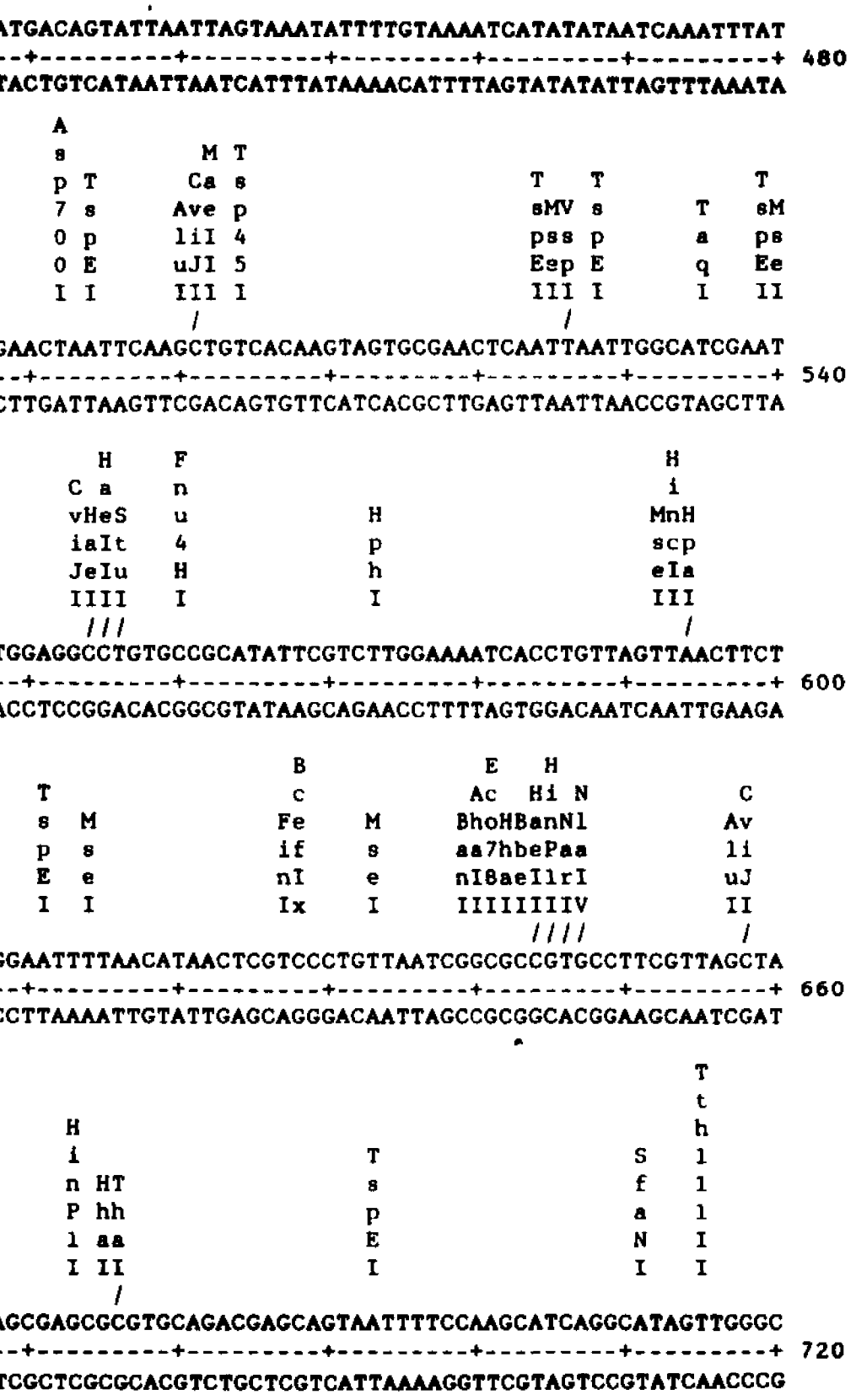
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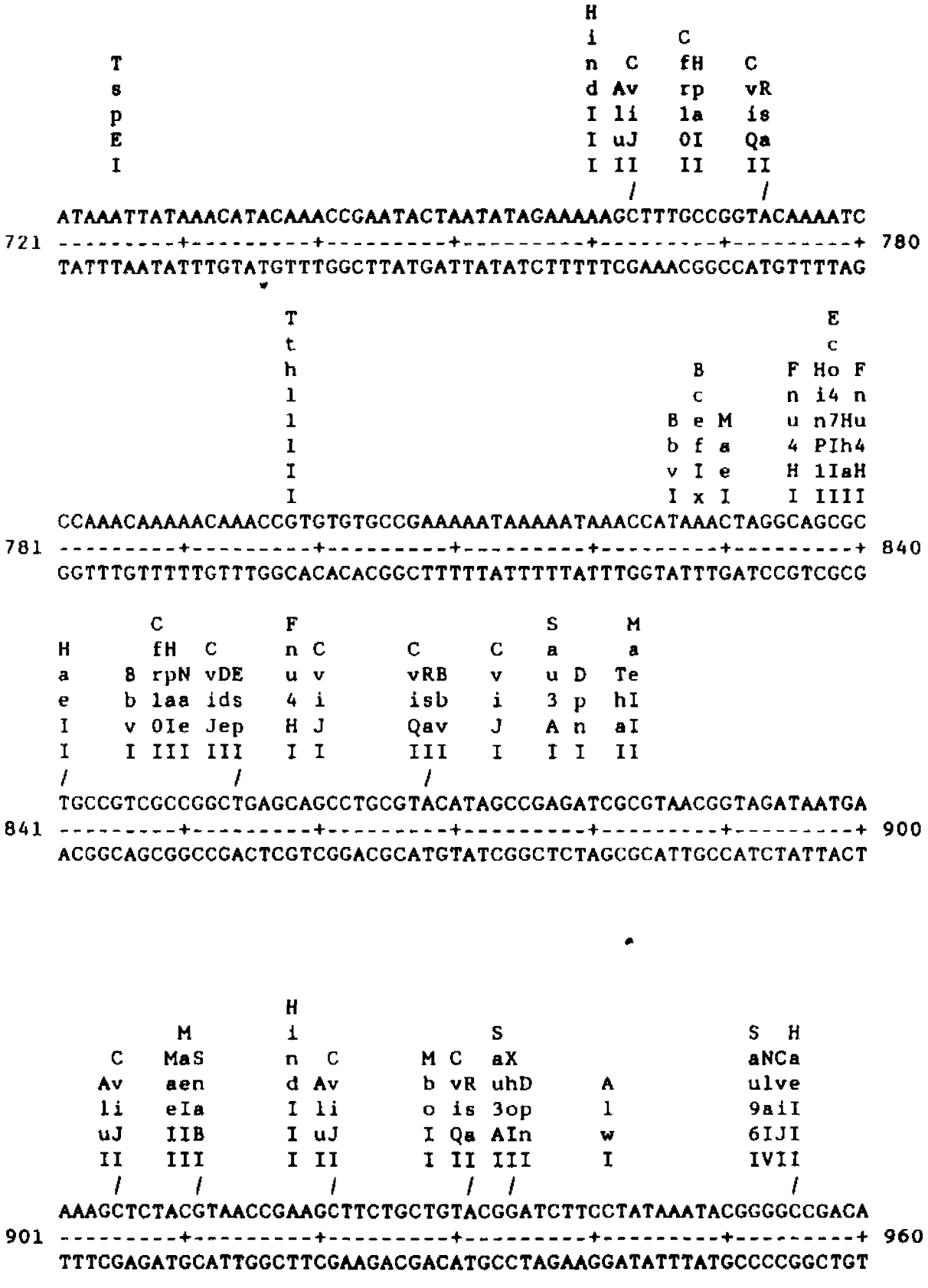
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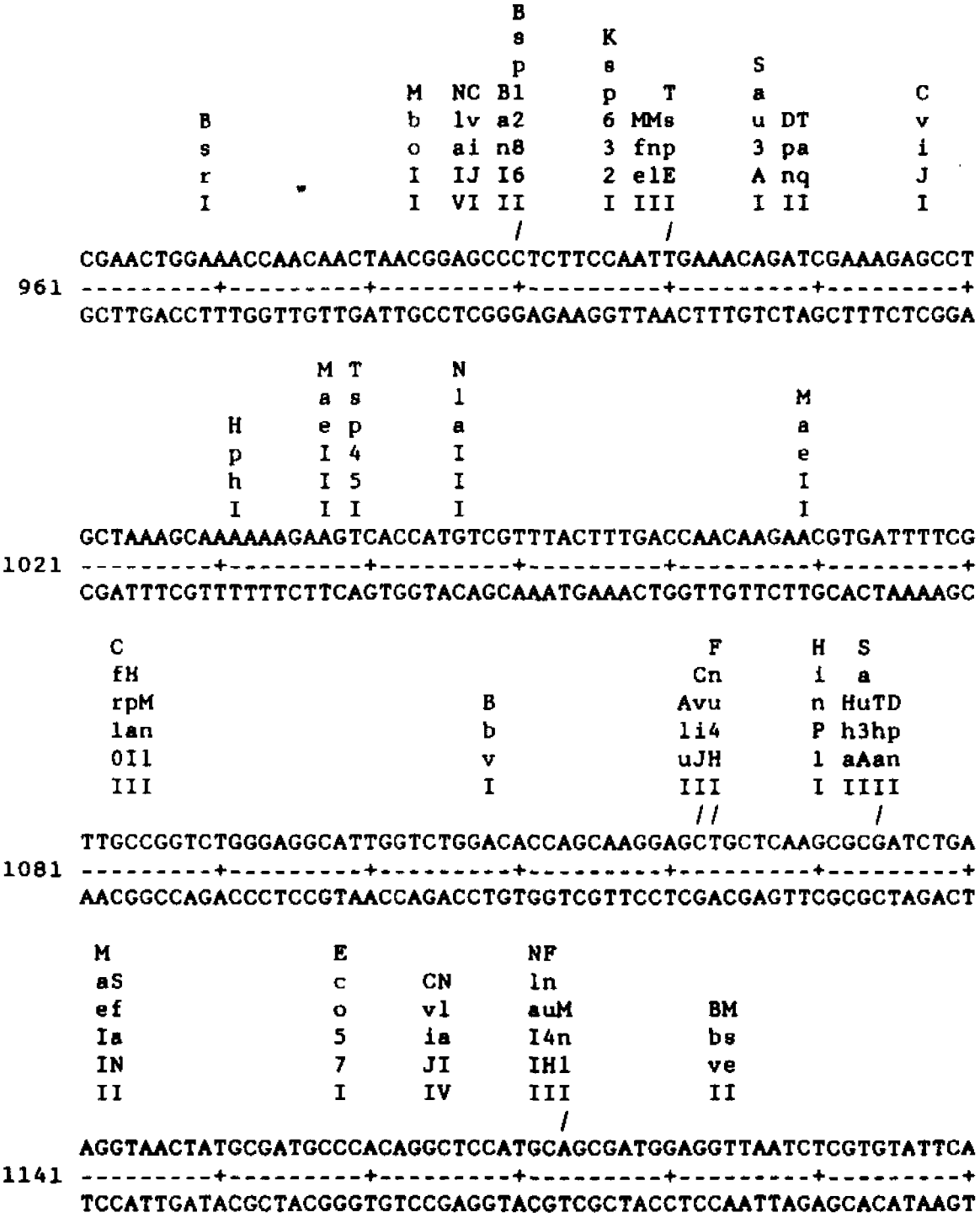
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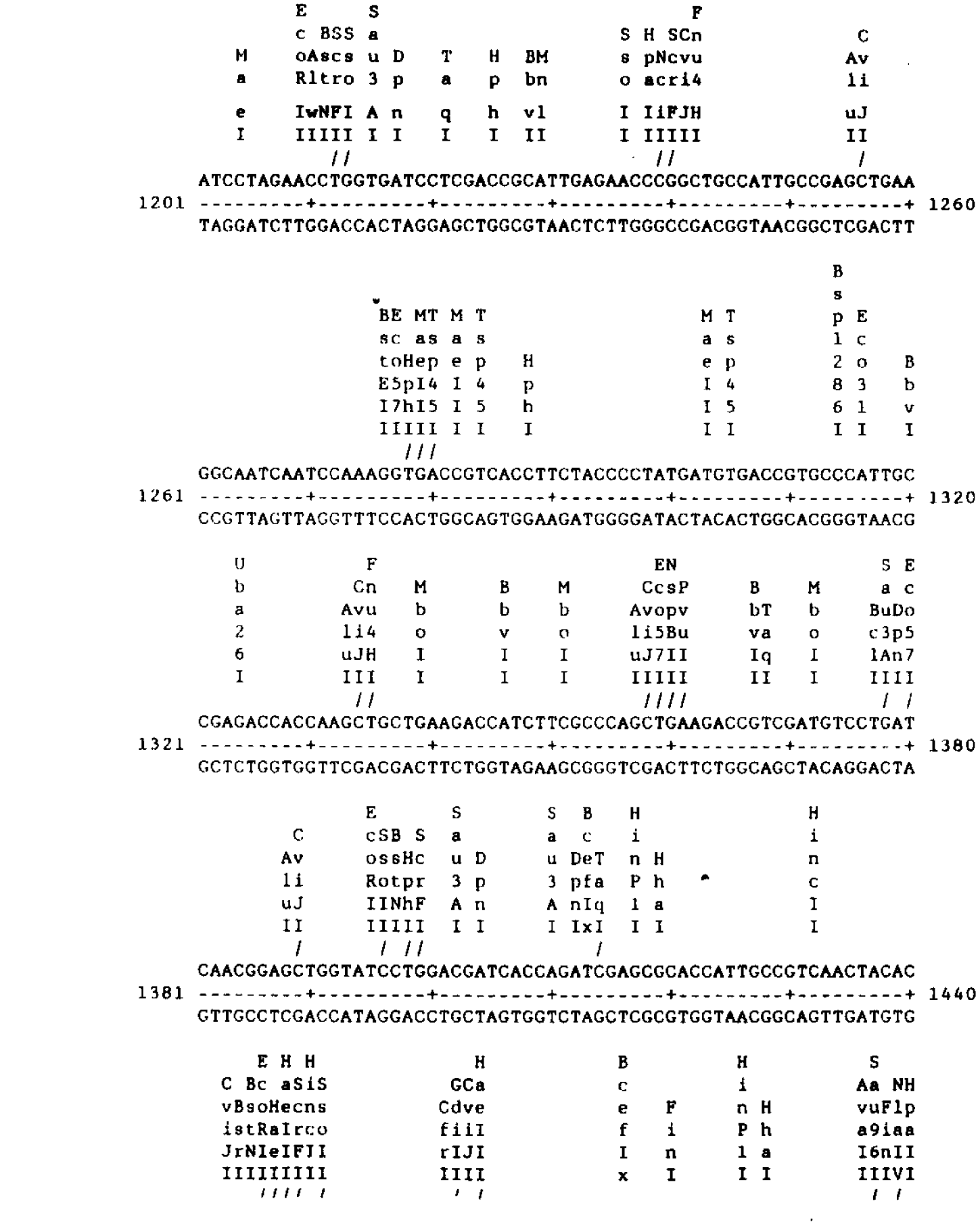
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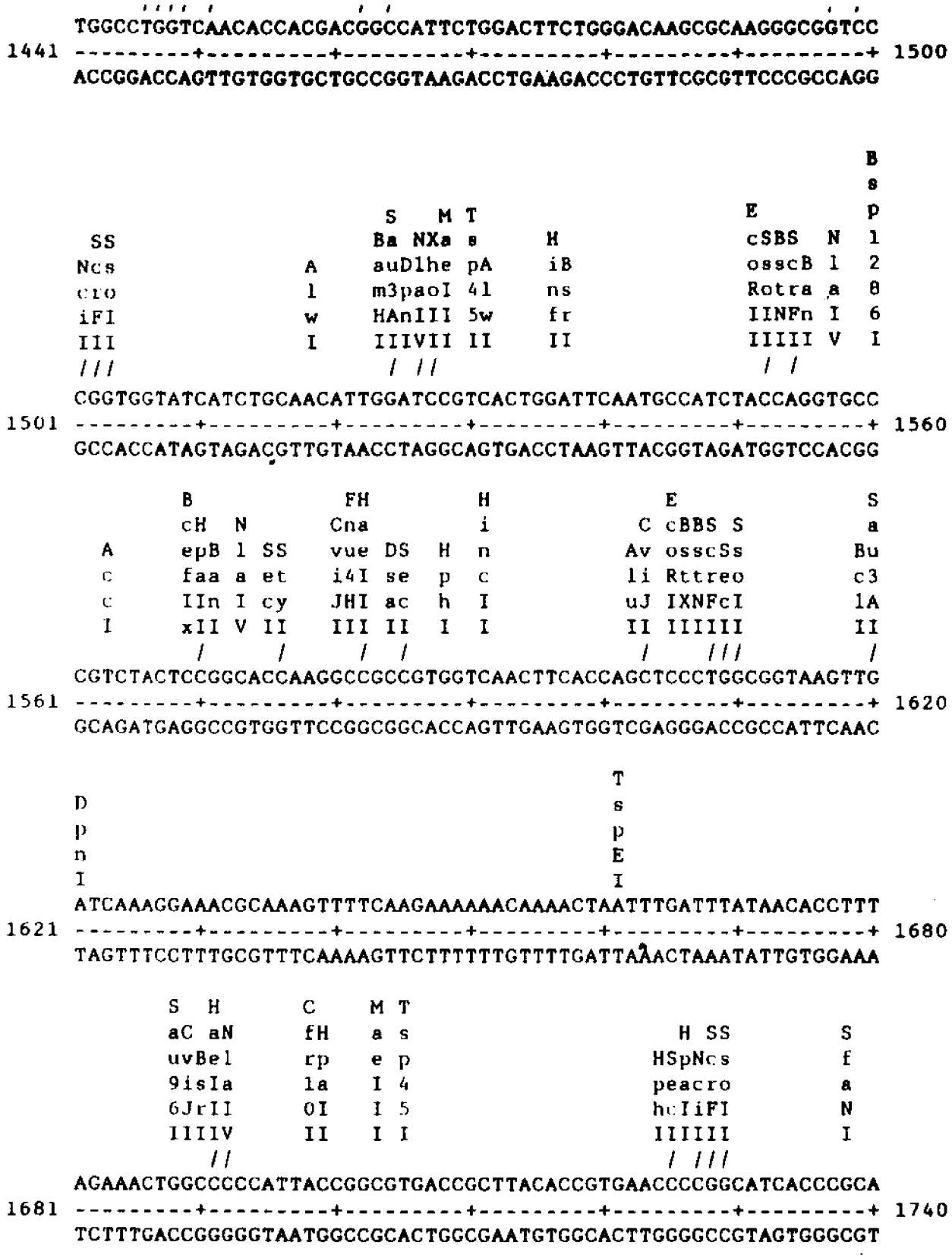
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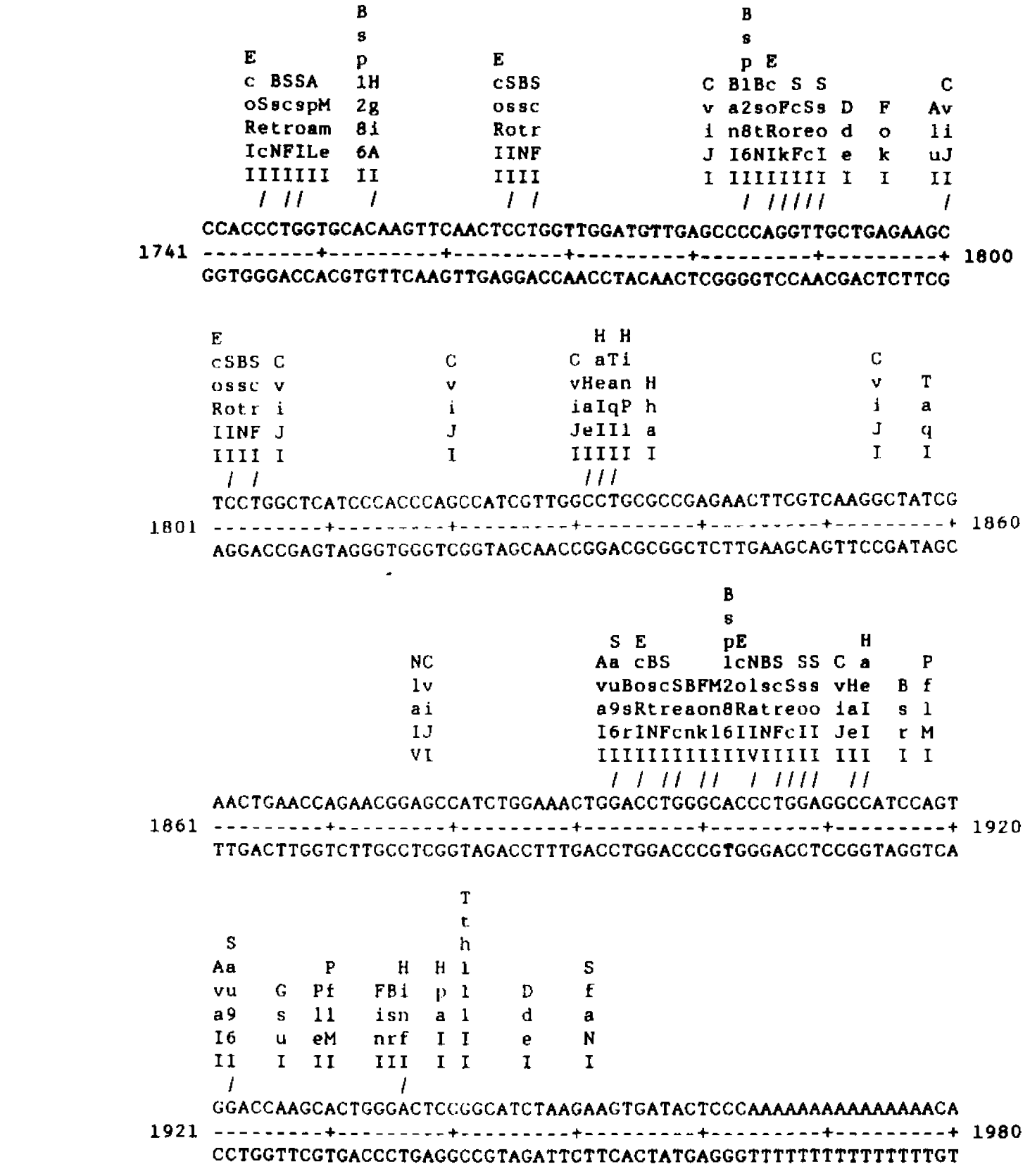
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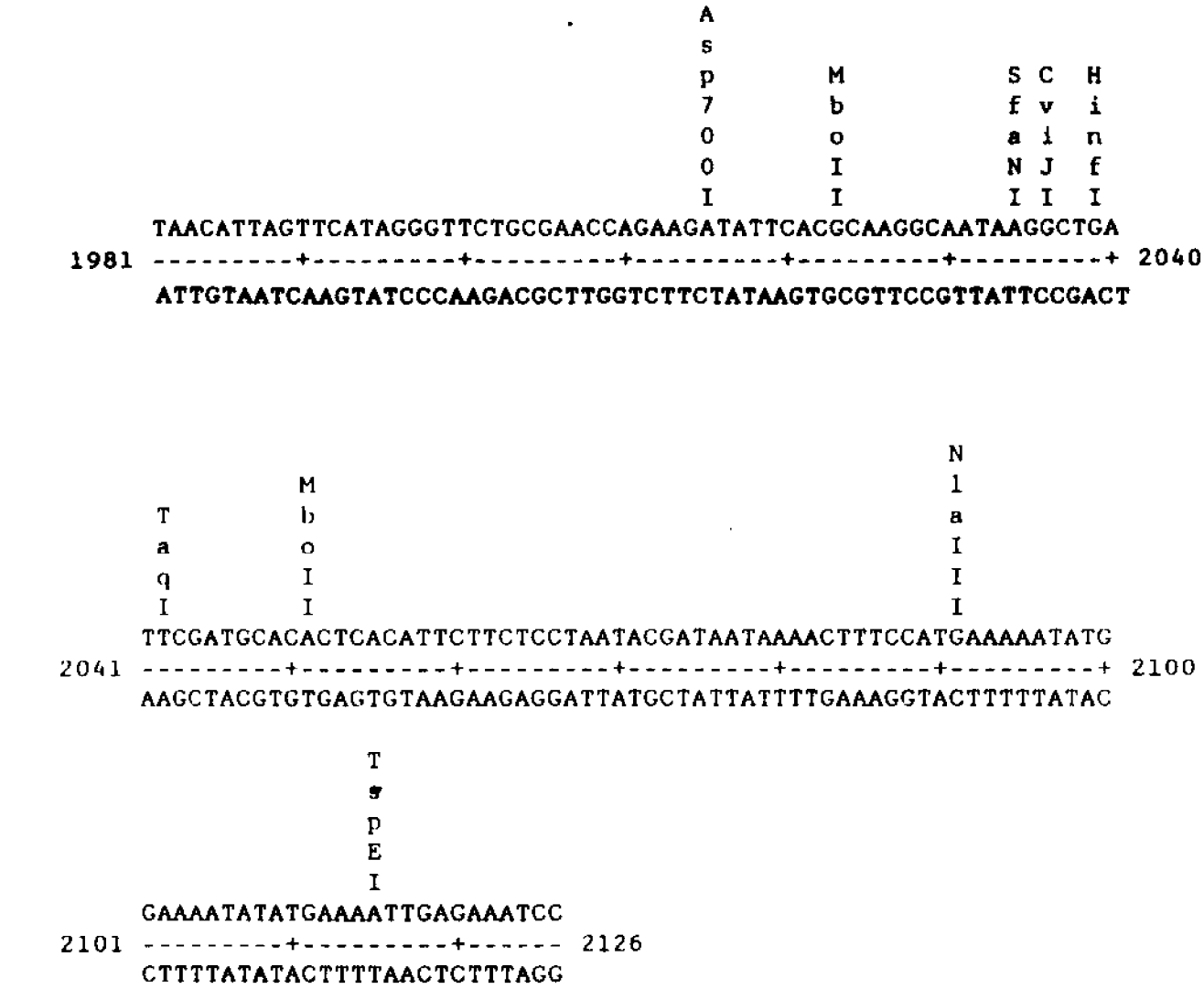

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BssHII	ClaI	DraI	DraII	DraIII	EcoNI	EcoRI	EcoRV
HgaI	HgiEII	KpnI	MluI	NcoI	NdeI	NheI	NotI
NruI	NsiI	NspHI	PmaCI	PpuMI	PssI	PstI	PvuI
RsrII	SacI	SacII	SciI	SfiI	SmaI	SpeI	SphI
SplI	Tth111I	XbaI	XcaI	XhoI	XmaI	XmaIII	

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